# Comparison of four tumor markers at the RNA and protein level for the detection of micrometastases and disseminated tumor cells in lymph nodes of patients with cervical carcinoma

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# Abstract

Lymph node status is the key prognostic factor for disease recurrence and patient mortality among cervical cancer patients. Patients with positive lymph nodes (pN1) have a high risk for recurrence. However, 15% of treated patients suffer recurrent disease although their lymph nodes have no evident metastases or micrometastases (pN0). The presence of occult tumor cells or tumor cell clusters smaller than micrometastases (<0.2mm) in lymph nodes could be the reason for poor prognosis of these patients. The goal of this dissertation is the detection of occult tumor cells and clusters in lymph nodes by using different molecular tumor markers and the measurement of the reliability on each marker by comparing them to each other. A highly sensitive, and at the same time, specific detection of these tumor cells is a prerequisite for further research confirming the clinical importance of occult tumor cells in lymph nodes.

In this study, immunohistochemical staining (IHC) and reverse transcription nested PCR (RT-PCR) were used to detect metastatic tumor cells or clusters in sentinel lymph nodes (SLN) of 48 patients with primary cervical cancer. 120 lymph nodes were evaluated. IHC was performed with a pan-reactive antibody against cytokeratins (AE1/3), an antibody against CK19 and an antibody against $p16^{INK4a}$. The latter protein, which is a surrogate marker of viral oncogene activity, is invariably upregulated in cervical cancers. Moreover, the viral oncogene (HPV-mRNA) activity was directly detected by RT-PCR.

First, the applicability of the immunohistochemical markers was validated by using 35 pN1 lymph nodes. Expression of the CK19 was inconsistent. Metastases or micrometastases of some lymph nodes showed a heterogeneous staining or completely failed to stain. On the other hand, the markers AE1/3 and $p16^{INK4a}$ stained homogeneously. Subsequently, 85 pN0 lymph nodes were evaluated for possible presence of tumor cells. To achieve that, the existing TNM classification of AJCC was extended to capture occult tumor cells or tumor cell clusters as well: Group A contains lymph nodes with metastases or micrometastases larger than or equal to 0.2mm. Based on the number of cells per microscopic field ($20^{x}$), single tumor cells or tumor cell clusters are classified into the Group B (more than 10 cells per field), Group C (less than 10 cells per field) and Group D (single isolated tumor cells per field). This classification allows for a statistical comparison of markers with the help of Cohen's Kappa Statistics and the "two by two" tables of agreement. For this comparison, AE1/3 was used as a gold standard. The statistical correlation between the IHC markers provided a "perfect agreement" of the markers AE1/3 and $p16^{INK4a}$ for the detection of metastases and micrometastases (Group A). However, a considerable discordance was determined

during the correlation measurement of IHC markers for the detection of occult tumor cells or clusters (Groups B, C, and D). The Cohen's Kappa Statistic showed only a "fair agreement" between AE1/3 and $p16^{INK4a}$ for the group combinations AB, ABC and ABCD. The correlation between AE1/3 and CK19 showed a "considerable agreement" for the Group AB, but only a "fair agreement" for the Groups ABC and ABCD. The reason for the bad results during the application of markers for the Groups B, C and D are the blurred borders between these three Groups caused by multiple sectioning. Furthermore, in some cases the differentiation between staining artifacts and single isolated tumor cells was hardly possible. This resulted in a "poor agreement" of IHC markers at the single cell level (Group D). It is therefore reasonable to consider only the Group ABC for the further marker evaluation.

Some discordances were also observed when comparing IHC and the HPV mRNA markers. These discordances were caused by the evaluation of different regions of lymph node tissue with IHC and RT-PCR methods. In some cases, the tumor was present either in the part of lymph node used for RT-PCR or in the one used for IHC staining. The RT-PCR method detects the presence of HPV mRNA in each tumor cell. The result of the detection (positive or negative) was compared with the results of IHC markers by the help of statistical methods. As expected, HPV mRNA provided the best (but only "fair") agreement with the HPV HPV-surrogate marker $p16^{INK4a}$.

This pilot study confirmed that a single marker is hardly appropriate for a reliable detection of occult tumor cells. Due to the non-random distribution of tumor cells in lymph nodes, multiple sectioning is required to achieve higher sensitivity. The sectioning has to be performed representatively providing the biggest possible longitudinal sections. In this regard, molecular markers acting at the RNA level provide an obvious advantage. A verification of the reliability of $p16^{INK4a}$ and AE1/3 for occult tumor cells or clusters in lymph nodes requires larger studies with more patients and a bigger sample size.

# Kurzfassung

Der Lymphknotenstatus stellt den wichtigsten prognostischen Faktor für Patientinnen mit primärem Gebärmutterhalskrebs dar. Patientinnen mit befallenen Lymphknoten (pN1) haben ein hohes Risiko ein Rezidiv zu entwickeln. Allerdings erleiden auch 15% der behandelten Patienten deren Lymphknoten keine Metastasen oder Mikrometastasen aufweisen (pN0), ein Rezidiv. Der Grund für die schlechte Prognose dieser Patientinnen könnten okkulte Tumorzellen oder Tumorzellcluster (kleiner als 0.2mm) in den Lymphknoten sein. Ziel dieser Doktorarbeit ist okkulte Tumorzellen bzw. -cluster mit Hilfe verschiedener molekularer Markern in Lymphknoten nachzuweisen und die Wertigkeit der einzelnen Marker im Vergleich zu bestimmen. Ein hoch sensitiver und zugleich spezifischer Nachweis dieser Tumorzellen stellt eine Voraussetzung dar, um die klinische Bedeutung okkulter Tumorzellen in Lymphknoten in nachfolgenden Studien zu untersuchen.

In dieser Studie wurden die im Abflussgebiet der Lymphflüssigkeit eines Tumors an erster Stelle liegenden und somit einem höheren Befallrisiko ausgesetzten Lymphknoten (Wächterlymphknoten) von 48 Frauen mit primärem Gebärmutterhalskrebs evaluiert. Dabei wurden 120 Wächterlymphknoten (davon 85 pN0) sowohl immunohistochemisch (IHC) als auch durch RT-PCR untersucht. Für die IHC-Analyse wurden ein pan-reaktiver Antikörper gegen Cytokeratine (AE1/3), ein Antikörper gegen Cytokeratin 19 (CK19) und ein Antikörper gegen $p16^{INK4a}$ eingesetzt. Bei der RT-PCR-Analyse wurde HPV mRNA als Marker detektiert.

Zunächst wurde die Eignung der eingesetzten immunohistochemischen Marker anhand von 35 pN1-Lymphknoten getestet. Es wurde festgestellt, dass die Expression des CK19-Markers großen Schwankungen unterlag. So wiesen Metastasen bzw. Mikrometastasen einiger Lymphknoten eine heterogene Färbung auf bzw. wurden gar nicht gefärbt. Die Marker AE1/3 und $p16^{INK4a}$ hingegen zeigten eine homogene Färbung.

Die 85 pN0-Lymphknoten wurden nun hinsichtlich eines möglichen Tumorzellbefalls untersucht. Dazu wurde die von der AJCC etablierte TNM-Klassifikation modifiziert um auch okkulte Tumorzellen bzw. –cluster zu erfassen: Gruppe A umfasst Lymphknoten mit Metastasen bzw. Mikrometastasen größer als 0.2mm. Einzelne Tumorzellen bzw. Tumorzellcluster werden in Abhängigkeit ihrer Anzahl in die Gruppe B (mehr als 10), C (weniger als 10) bzw. D (einzelne isolierte Zellen) pro mikroskopische Feld ($20^{x}$) eingeordnet.

Diese Klassifikation ermöglicht nun eine vergleichende Analyse der Marker mit Hilfe einer Cohens-Kappa-Statistik bzw. den 2-mal-2-Tafeln. Dabei wurde AE1/3 als Goldstandard

verwendet. Eine statistische Korrelationsmessung zwischen den IHC-Markern AE1/3, CK19 und $p16^{INK4a}$ hat eine perfekte Übereinstimmung der Marker AE1/3 und $p16^{INK4a}$ für den Nachweis von Metastasen oder Mikrometastasen (Gruppe A) ergeben. Eine bedeutende Diskordanz wurde jedoch bei der Korrelationsmessung der IHC-Marker für die Detektion okkulter Tumorzellen bzw. –cluster (Gruppen B, C, D) festgestellt. Zwischen AE1/3 und $p16^{INK4a}$ hat die Cohens-Kappa-Statistik nur eine „ausreichende" Übereinstimmung für die Gruppen AB, ABC und ABCD ergeben. Die Korrelation zwischen AE1/3 und CK19 zeigte eine „beträchtliche" Übereinstimmung für die Gruppe AB aber nur eine „ausreichende" Übereinstimmung für die Gruppen ABC und ABCD. Die schlechteren Ergebnisse bei der Anwendung der Marker für die Gruppen B, C und D sind auf die verschwimmenden Grenzen zwischen diesen Gruppen bedingt durch multiple Schnitte zurückzuführen. Des Weiteren war es in einigen Fällen schwierig zwischen Färbeartefakten und einzelnen isolierten Tumorzellen zu differenzieren. Dies führt zu einer „schlechten" Übereinstimmung der IHC-Marker auf Einzelzellenniveau (Gruppe D). Aus den oben genannten Gründen erscheint es sinnvoll, ausschließlich die kombinierte Gruppe ABC für die weitere Marker-Evaluation zu betrachten.

Auch beim Vergleich der IHC-Marker mit HPV mRNA wurden Diskordanzen festgestellt. Diese sind durch die Evaluierung der verschiedenen Regionen des Lymphknotengewebes durch die Anwendung der IHC- sowie der RT-PCR-Methoden bedingt. Bei der RT-PCR-Methode wird die Präsenz der HPV mRNA, die in jeder Tumorzelle vorliegt, nachgewiesen. Dafür ist eine Gesamtanalyse mehrerer Schnitte erforderlich. Das Ergebnis der Überprüfung (positiv bzw. negativ) wurde mit statistischen Methoden mit den Ergebnissen der IHC-Marker verglichen. Dabei lieferte HPV mRNA, wie erwartet, die beste (jedoch nur „ausreichende") Übereinstimmung mit dem HPV-Surrogatmarker $p16^{INK4a}$.

Diese Pilotstudie hat gezeigt, dass ein einzelner Marker für eine zuverlässige Detektion von okkulten Tumorzellen weniger geeignet ist. Es ist weiterhin anzumerken, dass aufgrund einer Nichtzufallsverteilung der Tumorzellen in Lymphknoten multiple Schnitte benötigt werden, um eine höhere Sensitivität zu erreichen. Die Schnitte müssen dabei „repräsentativ" sein, d.h. einen möglichst großen Gewebe-Längsschnitt bilden. In dieser Hinsicht bieten die molekularen Marker auf RNA-Ebene einen klaren Vorteil. Um zu überprüfen, ob $p16^{INK4a}$ und AE1/3 zuverlässige Marker für okkulte Tumorzellen bzw. -cluster in Lymphknoten sind, sind größere Studien mit mehr Patientinnen und einem größerem Probenumfang durchzuführen.

# Abbreviations

| | |
|---|---|
| AE1/3 | Cytokeratin cocktail AE1 and AE3 |
| AJCC | American Joint Committee on Cancer |
| ASCUS | Atypical Squamous Cells of Undetermined Significance |
| CCa | Cervical Cancer |
| CIN | Cervical Intraepithelial Neoplasia |
| CK19 | Cytokeratin 19 |
| CMI | Cell-mediated immunity |
| DNA | Deoxyribonucleic Acid |
| ER | Early Region |
| ERS | Epitope Retrieval Solution |
| FIGO | Federation Internationale de Gynecologie et d'Obstetrique (International Federation of Genecology and Obstetrics) |
| H-E | Immunohistochemical staining with Hematoxylin – Eosin |
| HEV | High Endothelial Venules |
| HPV | Human Papilloma Virus |
| HR-HPV | High Risk Human Papilloma Virus |
| IHC | Immunohistochemistry |
| INF | Interferon |
| LN | Lymph Node |
| LR | Late Region |
| LVSI | Lymph-Vascular Space Involvement |
| mRNA | messenger Ribonucleic Acid |
| MHC | Major Compatibility Complex |
| MIS | Minimally Invasive Surgery |
| NK cells | Natural Killer cells |
| NPV | Negative Predictive Value |
| ORF | Open Reading Frames |
| p16 | HPV surrogate marker $p16^{INK4a}$ |
| PCR | Polymerase Chain Reaction |
| PDZ | Post Synaptic Density Protein |
| pRB | Retinoblastoma Protein |
| RT-PCR | Real Time Polymerase Chain Reaction |
| SLN | Sentinel Lymph Node |
| tRNA | Transfer Ribonucleic Acid |
| TCR | T-cell receptors |
| TNM | A classification that stays for: tumor (T), node (N) and metastase (M) |
| TSG | Tumor Suppressor Genes |
| UICC | International Union Against Cancer |
| URR | Upper Regulatory Region |

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# Chapter 1: Introduction

Tumors of cervix uteri are mainly squamous cell carcinomas or adenocarcinomas. Rare types of cervical cancer (CCa) such as adenosquamous, papillary, villoglandular, anaplastic, transitional, spindle, adenoid basal, colloid, neuroendocrine, and glassy cell carcinomas represent only less than 5% of all cervical carcinomas. This chapter will briefly introduce the causal factors and mechanisms of CCa, their association with the human papilloma virus (HPV), which is present in almost all cervical carcinomas (Matthews-Greer et al. 2004), as well as the mechanisms of cancer progression and spread.

Tumor cells can escape from the primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and metastasize in a distant tissue somewhere else in the body. The frequent disease recurrence after patients with a primary cancer are treated might be caused by the presence of occult tumor cells or tumor cell clusters smaller than micrometastases or metastases and thus ignored by the conventional histology. These cells can be detected by using tumor-specific markers. Tumor markers for lymph nodes (LN) are, however, still under investigation. This study compares four potential markers for disseminated tumor cells in LN. These markers are also described in this chapter.

## 1.1 Cervical Cancer

Approximately 350,000 women, at a median age of 52.2, die every year from CCa worldwide, and there are about 500,000 women newly diagnosed with the disease every year (WHO 2002). Although CCa is considered a preventable disease, it is the second most frequent neoplasm in women worldwide. Generally,

cervical carcinoma develops no symptoms. Therefore, the best way to detect cervical cancer at an early stage is the participation of women in screening programs (DKG 2006, Rieck and Fiander 2006, zur Hausen 2002). The cytodiagnostic Papanicolaou test (Pap smear) is a necessary test to detect early CCa, or changes in the cells that could become cancerous. It identifies abnormal alterations of the cervical mucosa by microscopically evaluating the cells taken from the cervix uteri. The test was developed by the Greek-American cytologist and pathologist, George Papanicolaou (Papanicolaou and Traut 1997). The Pap test has successfully contributed to an overall decrease in the incidence of CCa. However, it does have its limitations that result from the subjective morphological criteria used to identify and categorize tumor cells (Franco 2003, Vassilakos et al. 1997). Moreover, the success of Pap test relies also on the woman herself. According to the American Cancer Society, the woman should undergo the Pap test regularly, about three years after becoming sexually active and no later than 21 year (ACS 2009, Cox 2003). Sampling errors might cause false-negative reports in cases where smears did not have sufficient evidence for diagnosis. For this reason, the Pap test does not provide an ideal sensitivity and specificity. Pap test results are scored abnormal when it detects non-cancerous (benign), precancerous (some abnormal cell changes) or malignant (possibly cancerous) cells. When the Pap test result is abnormal, a repeated Pap smear and a histological follow-up are recommended. If the test suggests a severe abnormality or cancer, then the colposcopical examination with biopsies is necessary. Biopsies are taken from different parts of the cervix or from the endocervical canal and the tissue is then microscopically examined for atypical cells. There is still a demand for new markers to improve the quality and accuracy of cervical screening and the triage of women with atypical Pap smears (ASCUS) (ACS 2009).

The incidence of CCa is seen to be higher in developing countries, where the socio-economical status is low and where the population-wide screening with the cytological Papanicolaou test is barely performed (Baak et al. 1995, Dürst et al. 2003). In Germany, for instance, the incidence of CCa is gradually decreasing: While in 1971, 35 among 100,000 women were CCa positive, in 1998 the incidence was 16.7 out of 100,000 women, and in 2001 the incidence was only 12 out of 100,000 women (Engel and Schubert-Fritschle 2004).

Cervical cancer is considered to be caused by the development and progression of milder epithelial disorders, so called “dysplasia” or “cervical intraepithelial neoplasia” (CIN). Nowadays, several factors are known to support and enhance the development of cervical uterine abnormalities, but research studies have highlighted HPV as the fundamental etiological factor for CCa (Brinton and Hoover 1997, Lynch et al. 1992, Schneider et al. 2001, Stern and Neely 1963, Walboomers and Meijer 1997, Wieland and Pfister 1997, Winkelstein 1990, Wolf et al. 1975, zur Hausen 1996, zur Hausen 2002). However, prospective data on the risks of cervical pre-cancer associated with specific HPV genotypes are limited.

### 1.1.1 CCa diagnosis and its correlation with the HPV infection

The correlation between human papillomavirus and cervical cancer was first suspected almost 25 years ago (Dürst et al. 1983, zur Hausen 2002). The advent of molecular cloning during the 1970’s led to resurgence in papillomavirus research. The unlimited availability of wild-type and mutant viral genomes made it possible to study the function of viral genes and their products, to use viral sequences as molecular probes, to detect papillomavirus sequences in tissue, and to identify and molecularly clone new viral genotypes. Application of these molecular techniques led to the identification of HPV as the necessary infectious cause of a major public health problem, cervical cancer. Nowadays, the role of HPV in the development of all types of cervical cancer, including also the rare types such as neuroendocrine or small cell carcinomas, is well established (Barrera et al. 2003, Bosch et al. 2002, Bosch et al. 2001, Matthews-Greer et al. 2004, Slattery et al. 1989, zur Hausen et al. 1974). A persistent infection with a high-risk HPV is a prerequisite for cervical carcinogenesis. A worldwide study of Walboomers and colleagues confirmed that 99.7% of all invasive cervical carcinomas are HPV DNA positive (Walboomers et al. 1999).

More than 100 genotypes of HPV are identified to date. They are classified in low-risk and high-risk viruses. The HPV types that have been classified as high-risk have gradually increased over time after their predisposition to cause pre-cancerous or cancerous lesions was evidenced (Muñoz et al. 2003). 15 HPV types are now classified as high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51,

52, 56, 58, 59, 68, 73 and 82); three are classified as probable high-risk types (HPV 26, 53 and 66); and 12 are classified as low risk types (HPV 6, 11, 40, 42, 43, 54, 61, 70, 72, 81, and CP6108). Low risk HPV are also called non-oncogenic types, as they do not associate with CCa, while the high risk HPV are called oncogenic types because they are present in almost all CCa (Wieland and Pfister 1997, zur Hausen 2002). From all high-risk HPV types, HPV16 has the most oncogenic potential and is responsible for 50% of cases of cervical cancer worldwide, followed by the HPV 18, 45 and 31. An evaluation of 85 studies that involved 10,058 women with squamous cell carcinoma and adeno or adenosquamous carcinoma showed that 51% of cases with invasive cervical cancer were associated with HPV16 and 16.2% with HPV18 infection. HPV16 had the highest prevalence among cases with squamous cell carcinoma (46-63% in all regions of the world), followed by HPV18 (10-14% in all regions except Asia). HPV18 was the predominant type in cases with adeno and adeno-squamous carcinoma in every region (37-41%) followed by HPV16 (26-36%) (Figure 1). More than 16 HPV types were associated with invasive cell carcinoma, collectively amounting to 18.3% of cases. However, the cases of this meta-analysis were not drawn uniformly from across each region; large areas have not been included, while specific populations such as Japan were overestimated. Furthermore, not all primer sets amplify individual HPV types with the same sensitivity, which is a potential source of bias in this analysis. In addition, many studies tested only for a subset of HPV types and did not consider potential multiple infections.

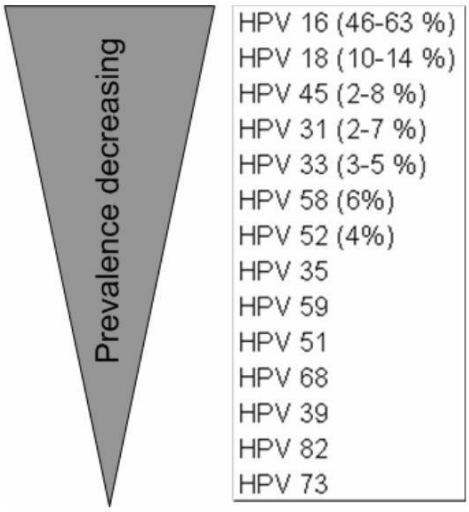



**Figure 1: Meta-analysis of overall HPV prevalence in squamous cell carcinoma (data taken from (Clifford et al. 2003))**

Although HPV infection is a necessary condition for malignant transformation, the process of malignant transformation requires co-factors. Some examples of risk factors contributing to CCa are reported in (Bosch et al. 2002, Bosch et al. 2001, Brinton and Hoover 1997, Dürst et al. 2003, Kjaer et al. 1997, Lynch et al. 1992, Schneider et al. 2001, Slattery et al. 1989, Winkelstein 1990) and are summarized in Figure 2.

**HPV associated risk factors**

**(HPV DNA positive women)**

- Increased number of sexual partners
- Increased frequency of intercourse
- Early age of first intercourse
- Poor pre- and postnatal care
- Sexual behavior of male partner
- Uterine cervical lacerations
- Genetic predisposition

**Additional risk factors**

- Smoking
- Use of oral contraceptives
- Age
- Infection with other sexually transmitted diseases
- Diseases affecting immune system
- Immunosuppressive drugs
- Low socio-economic status
- Lifestyle factors such as obesity mainly caused by high intake of animal fat
- Diets low in nutritional factors (ß-carotene or vitamin C)

**Figure 2: Established and possible co-factors in the etiology of cervical cancer**

Sexual behavior is the main determinant of oncogenic and non-oncogenic HPV infections. Furthermore, the population studied is very important. An example is the variation of HPV infections across the age groups (Herrero et al. 2005). It was observed that cervical dysplasia occurs more often among women in their 20's, carcinoma in situ among 25-35 year-old women and invasive cancer after the age

of 40 (Brinton and Hoover 1997). Cervical dysplasia is considered to be a disease that can be prevented by avoiding exposure to risk factors like multiple sexual partners, smoking or usage of contraceptives. Furthermore, early diagnosis by regular Pap test followed by the histopathologic examination of the biopsies taken during colposcopy examination as well as early treatment of benign and pre-cancer lesions prevents development of invasive cancer. In addition, the vaccine against HPV is now available. Because HPV infection is a prerequisite for cervical carcinogenesis, vaccination against HPV would be the best way for preventing CCa. Some types of HPV are known to cause malignant transformation of epithelial tumors, not only in anogenital tract but also in other organs such as respiratory tract, skin, etc. (Steger and Pfister 2002), which means that HPV research is important for the prevention of several types of cancer.

### 1.1.2 The role of lymphatic system in cervical cancer spread

The lymphatic system plays an essential role for the spread of cancer. It is as important as the other clinical-pathological parameters, such as clinical stage, tumor size, involvement of parametrial space, age and uterine body extension. Nowadays, it is known that the LN status and disease prognosis are closely related to each other (Delgado et al. 1990, Moehrle et al. 2004).

In 1894, Halsted introduced the importance of radical mastectomy for the treatment of breast cancer (Halsted 1894), which was a significant improvement for cancer treatment at an early stage through adequate surgery. Later on, further studies developed hypothesis regarding the role of lymphatic system in cancer spread (Gilchrist 1940, Zeidman and Buss 1954). The theory that metastasizing tumor cells in the lymph nodes could be the explanation for development of secondary tumors and is consequently the reason for tumor progression and dissemination progressively evolved (Scheungraber et al. 2002). Based on this theory, primary and secondary cancers started to be treated locally by lymphadenectomy; however the extensive removal of lymph nodes can cause side effects or complications such as lymphedema, voiding disorders, hemorrhage, serocele formation and reduced immune protection. In the routine, after surgical treatment, patients with metastasized lymph nodes undergo follow-

up treatment by radio or radio-chemotherapy. Also patients with tumor free lymph nodes (pN0) are overtreated and experience morbidity without survival benefit.

Since 1960, Gould and colleagues realized that there are some specific lymph nodes, referred to as “sentinel” lymph nodes (SLN) that play a special role in cancer prognosis (Gould et al. 1960). The sentinel lymph nodes are the first nodes that drain the lymph fluid; therefore, they have higher chances to harbor metastasizing tumor cells. Based on this theory the “sentinel lymph node concept” (SLN concept) was introduced. This concept was important for the cancer management, as only the selective LN, called “sentinels” would be removed. The SLN concept was first applied to patients with melanoma, a skin cancer that can be treated if diagnosed early but is life-threatening when the disease spreads beyond the primary tumor in the form of micrometastases or metastases. Regarding the SLN concept, the primary tumor drains metastatic tumor cells firstly into one or more sentinel lymph nodes and secondarily into the remaining regional LN. Therefore, if the SLN are micrometastases-free, the non-SLN are also expected to be micrometastases-free. Roderick and colleagues found that among patients with breast carcinoma and tumor-free SLN only 0.1% (1 out of 1087) showed involvement of a “non-sentinel” node (Turner et al. 1997). The prediction of metastasis-positive regional LN (non-sentinel) after SLN were metastasis-free is, however, still being studied for patients with cancers of other organs as well (Cochran et al. 2003). Before the SLN concept was introduced, all regional LN were removed by surgery from all patients including the ones with missing evidence for cancer spread. The accuracy of SLN concept for CCa is, however, still under evaluation (Altgassen et al. 2008, Altgassen et al. 2006, Altgassen et al. 2007).

Among others, Barranger et al. studied the histopathological validation of the SLN concept in cervical cancer patients by evaluating the biopsies of SLN and non-SLN, and found that the SLN concept procedure reliably predicts the metastatic status of the regional LN in patients with CCa (Barranger et al. 2004); whereas Marchiolè et al do not agree with the accuracy of the SLN concept for CCa (Marchiolè et al. 2004). After evaluating SLN and non-SLN of patients with early stage of CCa by multi-sectioning IHC, Marchiolè and colleagues found that three out of five patients with positive non-SLN were SLN negative. However, the suggestion of Marchiolè et al was only based on results from 29 patients with

early cervical carcinoma, and they were using a staining substance (patent blue dye) alone, whereas using a combination of two staining substances promises higher SLN detection rate. Furthermore, the false negative rate of the SLN mapping could also be influenced by other parameters, such as mistakes during injection of labeling substance, surgery and IHC procedure. In 2008, the hypothesis-based, prospective multicenter cohort study of Altgassen and colleagues, evaluated immunohistochemically the lymph nodes of 590 patients with CCa. Detection rate, sensitivity, and negative predictive value (NPV) were calculated. This study showed that the overall sensitivity of the sentinel concept was low (77.4 %), which is lower than the predefined noninferiority margin (90 %); however, patients with tumor diameter smaller or equal to 2cm might profit from the SLN concept (Altgassen et al. 2008). If the SLN concept would be valid for CCa, than the removal and evaluation of only “sentinel” lymph nodes served for deciding on the appropriate treatment and further management of the patients with CCa. Although preliminary opinions suggest that the SLN concept can be offered to patients with tumors with diameter less than or equal to 2cm, this concept can still only be used in research studies (Altgassen et al. 2008, Altgassen et al. 2006, Schneider 2007). The SLN concept is until now proved to be valid for patients with melanoma and breast cancer (Moehrle et al. 2004). Studies are conducted to investigate whether this concept is valid for other organs such as carcinomas of head and neck or cancer of prostate (Begum et al. 2003, Kampen et al. 2006, Wawroschek et al. 1999).

As a general rule, disease recurrence is expected from lymph-node metastasis positive patients (pN1 status); patients with micrometastases-free LN (pN0 status) should have the best prognosis and disease survival (Lambert et al. 2006, Yuan et al. 1999). However, although LN are micrometastases-free, only 85.6% of patients have good prognosis during the 3-year disease-free interval (after surgical treatment with radical hysterectomy and pelvic lymphadenectomy). Disease recurrence occurs in 14.6% of the pN0 patients (Delgado et al. 1990). The reason for bad prognosis of these patients is still being discussed.

According to the American Joint Committee for Cancer Staging (AJCC) for Breast Cancer, micrometastases are considered to be tumors larger than 0.2mm, but smaller than 2mm (AJCC 2002b, Singletary et al. 2002, Singletary and Greene 2003). In this classification, the role of sporadic and isolated occult tumor cells or

clusters with dimensions smaller than 0.2mm is not taken into consideration. Furthermore, the conventional histopathological examination of the LN only detects cluster micrometastases larger than or equal to 0.2mm, while single tumor cells or tumor cell deposits, cannot be identified. These tumor cells could be, however, the reason for poor prognosis and increasing risk of recurrence for patients with cervical cancer after treatment. Therefore, in this study we have extended the micrometastases-staging of AJCC by also considering tumors smaller than 0.2mm. Apart from tumor clusters, isolated sporadic tumor cells and tumor cell deposits are taken into consideration. Accordingly, we defined four positive groups: A, B, C and D (explained and illustrated in Paragraph 3.2).

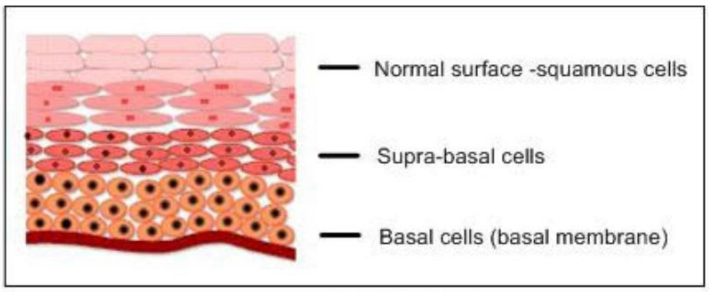
### 1.1.3 HPV infection in cervical epithelial and mucosal cells

The human papillomavirus is an oncogenic DNA virus that infects epithelial or mucosal cells. Following a multi-step process that involves a number of genetic changes in somatic cells it leads to cancer. The life cycle of HPV is strictly tied to epithelial differentiation (Doorbar 2006, Sausville 1992, Wawroschek et al. 1999). The virus enters the human body through scratches, wounds or mucosa. The early infection starts at the deeper basal or parabasal cell layers (Figure 3), which are the only dividing cells of the epithelium. The infection is usually self-limited; it can regress due to the immune response of the organism (Paragraph 1.1.3) (O'Brien and Campo 2003). The host cell requires a certain degree of terminal differentiation to be able to replicate the viral genomes. During the early infection there is only a minor viral gene expression and replication; the virus does not destroy the host cell. Only about 50 genome copies are generated once the host cell is divided into daughter cells. However, this situation changes when the infected epithelial cells reach terminal differentiation and the capacity to proliferate is irreversibly lost. In this condition, the viral genes may be strongly expressed and the replication cycle of the virus initiates until mature viral capsids are released. The transformation of epithelial cells is possible only in persisting infections, when molecular mechanisms that prevent the expression of viral genes in the immature basal and parabasal cells are lost. At this moment, the interference of viral genes with controllers of immature epithelial cell replication and life cycle may result in chromosomal instability. Deregulated expression of E6

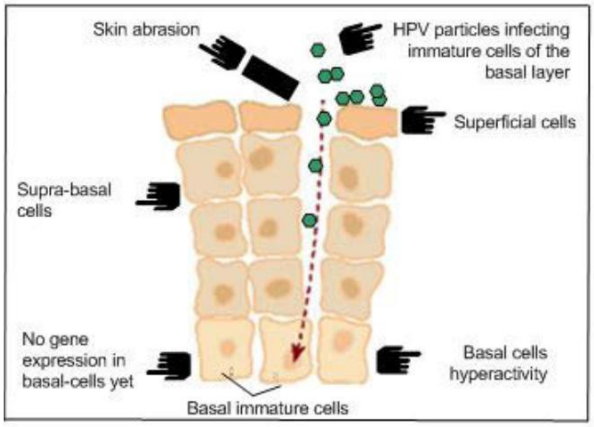
and E7 proteins in the dividing basal cells represents the first step in the multi-step process of HPV-mediated chromosomal instability (Doorbar 2006, Snijders et al. 2006). In essence, these viral proteins immortalize the human keratinocytes and drive cell proliferation through their association with PDZ[1] domain proteins and Rb (retinoblastoma) contributing to neoplastic progression. Three major aspects are involved in chromosomal instability (Stanley 2006):

- The E6 protein of the high-risk HPV types (HR-HPV) supports the premature degradation of the p53 tumor suppressor gene and, thus, interferes with normal apoptotic functions of epithelial cervical cells. It prevents the normal repair or chance mutations in the cellular genome.
- The E7 protein induces inactivation of the Rb protein complex and, thus, allows the cell to evade cell cycle control through the pRB pathway.
- Both E6 and E7 genes induce substantial disturbances of the mitotic functions by interfering with centrosome synthesis and function that result in desegregation of the chromosomes during mitosis and numerical and structural chromosomal aberrations.

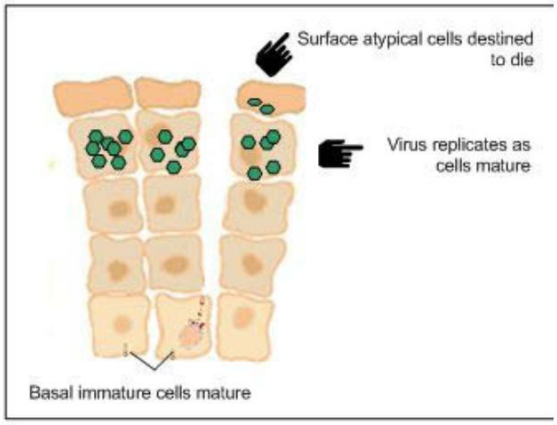
[1] PDZ is an acronym combining the first letters of three proteins: Postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occuldens-1 protein (zo-1) that were first discovered to share the domain.




(a) Normal epithelium




(b) Latent infection, no gene expression.




(c) Active and productive infection.

An HPV infection is restricted to the epithelium (a). The HPV life cycle differs from all other virus families; the infection begins in the basal keratinocytes layer (b) but the viral replication takes place in the upper layers of squamous epithelium (c) and not in the basal layer. There is only little or no viral protein expression in the deeper basal layers of the epithelium. Viral gene expression results in acanthosis, parakeratosis, and hyperkeratosis which then is clinically manifested as condylomata acuminata or genital warts. HPV can cause a productive infection where virions are produced or a non-productive infection where only some early genes are expressed but no viral production takes place. Productive infection does not lead to host cell lyses and the superficial keratinocytes containing the virus are eliminated by shedding.

**Figure 3: HPV infection in epithelial cells**

In summary, deregulated expressions of HPV E6, E7 and to lesser extent E5 oncogenes in the basal and parabasal cells can induce progression to cervical carcinomas. The interaction of oncoproteins encourages the abrogation of cell cycle control, chromosomal alterations, telomerase activation, and eventual cell immortalization. As the immature infected cells undergo mitotic defects and accumulate further genetic alterations, they can progress to invasive carcinoma (Barrasso and Guillemotonia 1997, Fehrmann and Laimins 2003).

#### 1.1.3.1 Process of HPV infection in the cervix

HPV infection follows a linear process. In the latent phase, the infection can be spontaneously eliminated due to the immune response of the organism. In this phase, the infection is asymptomatic but can be identified by molecular biology techniques. The process from viral infection to clinical appearance of warts takes at least 4-6 weeks (Stanley 2006); however in 80% of cases, the infection resolves due to body's immune response within 12 months (Weigel et al. 2006 ). In general, lack of immune cells is evidenced in non-regressing genital warts. CD4+ mediated mechanisms play a central role during the HPV infection. The insufficiency in CD4+ T-cells implies progression of HPV infection. This is the situation when genital warts don't regress. Progression of the latent HPV infection can manifest pre-neoplastic lesions that are called cervical intraepithelial neoplasia (CIN). Women who are HPV DNA positive for 18 months or longer have about 300 times higher risk than others to develop CIN (Bory et al. 2002). With regard to the progress of neoplasia, CIN lesions are classified as CIN1, CIN2 and CIN3. Also these lesions can spontaneously regress to the latent infection or to normal epithelium. CIN1 lesions are considered as low-risk neoplasia as they usually require a relatively long time before they progress to invasive cancer. CIN2 and CIN3 are considered as high-grade squamous intraepithelial lesions. About 20% of HR-HPV infected women will develop high-grade neoplasia within 4 to 36 months (Bory et al. 2002). Comparing to the latent infection and CIN 1, CIN2 and CIN3 have higher risk to progress to invasive cancer. CIN2, CIN3 and invasive cancer do not occur in absence of HR-HPV (Bosch et al. 2002, Walboomers and Meijer 1997). Persisting HR-HPV infection for years or decades leads to cervical cancer (Doorbar 2006). After malignant transformation, regressing to lower grades of neoplasia is not possible (Figure 4).

The long interval between the infection with HPV and the development of cervical cancer allows the screening programs using the Pap test to detect and treat CIN2/3 lesions prior to the development of CCa.

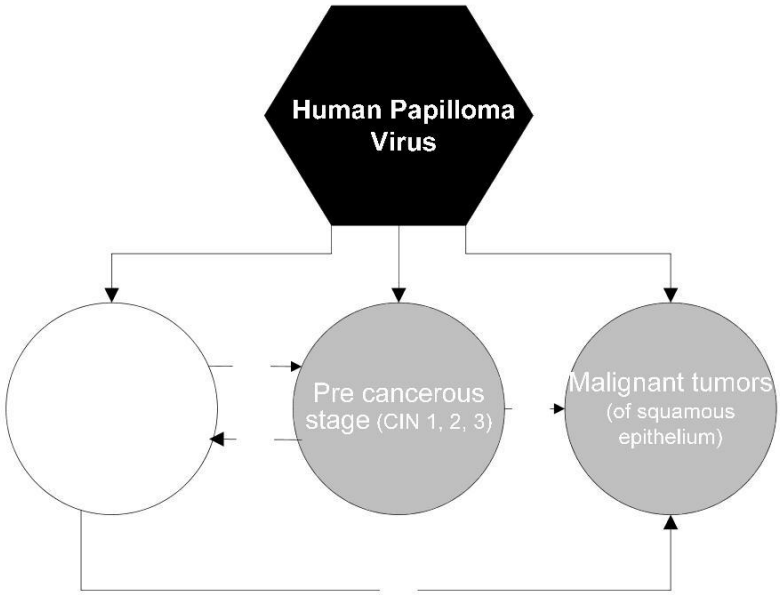



**Figure 4: Progression and regression ways of HPV induced neoplasia**

### 1.1.3.2 Immune response and regression mechanisms of HPV virus infection

Infection from HPV and vegetative viral growth is dependant on the differentiation program of the keratinocytes, the target squamous epithelial cells. Keratinocytes are destined to undergo terminal differentiation and desquamation. Therefore, inflammation is generally not associated with HPV infection. For this reason, limited or no immune response is expected. However, the human immune system involves a number of defense mechanisms against infection from HPV. Due to these mechanisms, genital warts often regress spontaneously (Stanley 2002). Persistent HPV infection is associated with lack of immune cells that are responsible for the defense from the virus. Consequently, the function of the immune system is weak. T-cells[2] are the main cells responsible for maximizing the function of immune system (O'Brien and Campo 2003). Antigen presence in the HPV induced warts stimulates T-cells to become either "cytotoxic" CD8+ cells or "helper" CD4+ cells. During the wart regression, massive mononuclear cells infiltrate both in stroma and in epithelium. The infiltrating lymphocytes are mainly CD4+, however, many CD8+ T-cells are present in the epithelium as well.

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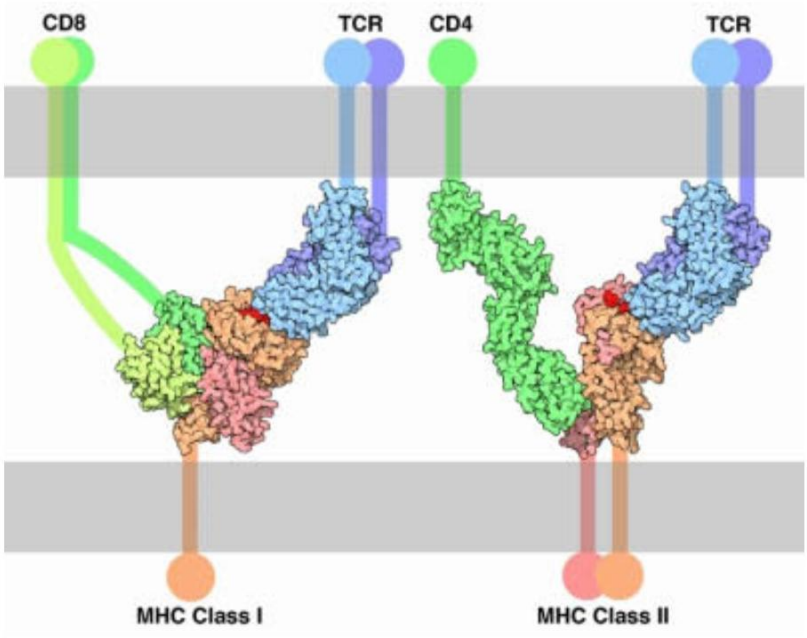
[2] "T" stands for thymus, the principal organ for development of T-cells. T-cells are lymphocytes and play a central role in cell-mediated immunity.




T-cell receptors (TCR) on the surface of T-cells bind tightly to viral peptides displayed in major histo-compatibility molecules (MHC). These molecules are synthesized within the endoplasmic reticulum, where they are exposed to peptide fragments of all proteins that are present in the cell. All proteins (including any viral peptide that is synthesized in the cell) are carried to the cell surface. Each T-cell has its own type of T-cell receptor, which recognizes its own type of peptide. T-cells bind to the T-cell receptors and CD8 or CD4 molecules. As T-cells mature, the immune system creates diverse T-cells, each with a different receptor, to protect from various types of infection. As a stem cell becomes a T-cell, it shuffles around its genes to form a random and unique T-cell receptor. Then, through a process of selection in the thymus, all T-cells that happen to recognize the normal proteins are destroyed, leaving only those that recognize foreign objects.

**Figure 5: Molecular association of CD8+ T-cells with MHC class I and CD4+ T-cells with MHC class II (PDB 2010)**

Observing how papillomavirus infects the epithelium and how the infection regresses was easier at animal models than in humans. Studying the infection of oral mucosa of canines with papillomavirus showed that there is a strong infiltrate of mononuclear cells in the wart one week before warts regression. CD4+ T-cells appear first in the wart and populate the stroma whereas at a later point in time and in smaller numbers CD8+ T-cells arrive in epithelium and stroma. Keratinocytes and endothelial cells of the small vessels of the wart-stroma are induced to express MHC Class II. Expression of MHC Class I is massively up-regulated, which is typical for a massive Cell-mediated immunity (CMI) response and the intracellular adhesion molecule 1. CMI is an immune response that does not involve antibodies but rather involves the activation of macrophages, NK

cells, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen (e.g. HPV). This means that a local release of cytokines such as INF-γ[3] happened (Figure 5). Lymphocyte tracking on the endothelium of warts´ capillaries requires up-regulation of the adhesion molecules. The local release of pro-inflammatory cytokines and chemokines is reinforced (Campbell et al. 2007, Campbell et al. 2000, Nicholls et al. 2001, Stanley 2006). E2 papillomavirus protein appears to be the target protein for this attack. Therefore, during different time points of the HPV infection cycle, systemic T-cell response directed to E2 peptides can be identified.

Contradictory, strong pro-inflammatory and anti-inflammatory responses can be detected in high-grade squamous intraepithelial lesions (HSIL). In particular, a strong Th1 response with abundant expression of INF-γ by CD4+; CD8+ and NK cells exists. Despite this, HR-HPV infected HSIL persist. Progression in HR-HPV-induced neoplasia is evidently accompanied by an increasingly immunosuppressive environment with the recruitment of regulatory T-cells and a cytokine environment in which IL-10 and TGF-β dominate. At the same time, responses to pro-inflammatory anti-viral cytokines such as INF-α and INF-β are lost, and the key immune response, including apoptosis and death of infected cells, are crippled (Stanley 2006). INF-α and INF-β can signal to directly prohibit viral metabolism, which is done by binding the INF receptors that signal for the synthesis of anti-viral proteins. The binding of INF to their receptors obtains multiple signal transduction pathways which lead to the activation of different sets of genes. When the cell is infected, the anti-viral proteins can bring it to a virus resistant status. INF-α and INF-β are potent inducers of NK cell-mediated cytotoxicity. In addition to INF-α and INF-β, a wide range of other innate cytokines can mediate biological functions regulating the NK cell responses of cytotoxicity, proliferation, and INF-γ production. When a virus infects an activated cell, the anti-virus proteins detect the foreign genetic material and block the viral replication by collapsing the mRNA and stopping the protein synthesis. These proteins bring the cell in stasis resulting in further replication of the virus together with distribution and dissemination of infection. Due to such holding action, the

[3] INF is a family of cytokines that exhibit a strong antiviral and anti-proliferating activity. When the genetic material of the virus enters the cell and starts to synthesize viral products, the cell is stimulated to produce type I (α and β) interferon. Type II interferon (INF γ) is produced by activated T-cells. It makes the cell easily recognizable by cytotoxic T-cells.

immune system has time to mobilize and activate the T-cell response (Roitt et al. 2001).

#### 1.1.3.3 Proto-oncogenes, oncogenes and tumor suppressor genes in dysplasia

The transforming genetic elements are named “proto-oncogenes” during their normal physiologic form and “oncogenes” when they contribute to malignant progression. Their activation plays an important role in completing the neoplastic phenotype. Several reasons such as exposure to radiation, chemicals or other carcinogens can induce oncogenesis. Oncogenes are dominant in their action; therefore, it is only necessary that one mutant gene-allele is involved in order to cause dysplasia. When proto-oncogenes mutate to become oncogenes, they maintain their functionality, but are no longer capable of responding to normal regulatory signals. As shown in Table 1, proto-oncogenes can be converted to oncogenes in the following three ways: Point mutation, gene amplification or chromosomal rearrangements (Stott and Wyse 2003).

**Table 1: Ways of mutating proto-oncogenes into oncogenes**

| | | |
|---|---|---|
| 1. | **Point mutation or deletion in coding sequence** | • Constitutively active protein is produced in normal amounts. |
| 2. | **Gene amplification** | • Normal protein is produced in much higher amounts. |
| 3. | **Chromosomal rearrangement** | • Assignment of strong enhancer, which causes overproduction of normal protein<br>• Fusion to another actively transcribed gene, which results in either increased level of the fusion product (normal activity, overproduced) or the fusion protein is hyperactive (increased activity in normal amounts) |

Usually, the cell division through mitosis is a finely regulated process that requires the involvement of one protein to activate another, which is known as a signal transduction cascade. This cascade finally concludes with changing the gene expression that prepares the cell for mitotic events. Oncogenes are important concerning the regulation of normal cell proliferation, differentiation, and programmed cell death. Thus, the molecular diagnosis and cancer monitoring are identified by confirming oncogene abnormalities. Many oncogenes were discovered in the last two decades, but, discovering of new oncogenes represents possible targets for innovative means of cancer treatments.

Furthermore, it offers new and improved ways for cancer diagnosis. Activated by dominant mutations, oncogenes promote cell growth, while tumor suppressors act in the normal cell as negative controllers of cell growth and are inactive in tumor cells. Tumor suppressor genes (TSG) are cellular genes that suppress cell growth and proliferation. They are associated with inhibition and cell division. TSG are usually deactivated by occurrence of mutations of their protein sequence (Pierotti et al. 2000). Because each allele of the gene acts as “recessive”, dysplasia occurs only when both alleles are altered. Tumor suppressor genes result from a mutation that is loosing the genetic function such as the point mutation or deletion in coding sequence (Table 1). Usually, loss of only one allele of the tumor suppressor gene is not sufficient to cause a disorder, given that the product of the normal allele remains to suppress cell growth.

#### 1.1.3.4 Molecular basis of HPV-related neoplasia

The genomic organization of HPV helps to understand the oncogenic process it induces to develop cervical dysplasia. Figure 6 illustrates the genomic organization of human papillomavirus (HPV16). Significant regions include Upper Regulatory Region (URR), Late Region (LR) and Early Region (ER). URR is responsible for the regulation of viral replication and controls the transcription of some sequences in the ER. LR encodes the structural proteins that are important for capsid production, which happen late in the viral life cycle. The infected basal cells, that show signs of cell deregulation as a result of the viral infection, continue their differentiation and migration to the epithelial surface, where the squamous cells start to express the late HPV genes L1 and L2. L1 encodes the capsid protein that makes 95% of the virions´ mass, whereas L2 encodes the minor capsid protein. ER encodes principally the proteins that are essential in viral DNA replication and gene expression, which occurs early in the viral life cycle. At the ER, the transformation and immortalization potency of HPV resides and consists of a number of genes that are regulatory for viral transcription and replication as well as cell cycle control. Viral proteins E1 and E2 are the major players in the control of replication and transcription, while E5 is the smallest oncogene protein consisting of 44 amino acids and segregating predominantly with cellular membranes (Schlegel et al. 1986). Open Reading Frames (ORF)

within the ER, code for proteins like E6 and E7 that are involved in the regulation of viral replication and the viral life cycle. Oncogenes E6 and E7 play the key role for the malignant transformation of the cells. In essence, they immortalize the human keratinocytes. Their respective oncoproteins are consistently overexpressed in malignant cells. E6 and E7 interact with cellular proteins implicated in the cell cycle control and consequently stimulate proliferation or intervene with the differentiation of papillomavirus infected cells. Oncoproteins are mitogenic and modulate apoptosis. Furthermore, by deregulating cell proliferation and originating genetic instability, oncoproteins encourage mutations that are essential for the acquisition of phenotypic transformations such as invasive growth, angiogenesis and metastasis (Bosch et al. 2001, Dürst et al. 2003, zur Hausen 2002).

E6 protein of oncogenic HPV types, that is located in the cell-nucleus, can induce the degradation of the anti-oncogenic regulator p53 either in vitro or in vivo, while E7 binds retinoblastoma gene products. Loss of p53 function is very important in the pathology of CCa. The sequence of events that disturb the normal apoptotic process of cervical cells, thus, transforming them into immortal dysplastic cells is as follows: circular DNA integrates via the E1/E2 genes; disruption of the E2 gene which is a negative regulator of E6 and E7 expression ensures constitutive viral oncogene expression; this leads to the inactivation of p53 and pRB which in turn leads to cell cycle progression and immortalization of normal cells because absence of functional p53 protein makes the cell extremely vulnerable to DNA damage and prevents the beginning of p53-mediated apoptosis. E6 protein of high risk HPV interacts with p53 and E6AP protein and leads towards protein p53 degradation, while the E6 protein of low-risk HPV types cannot target p53 for degradation. A C-terminal region among all HPV types is important for p53 binding; however, N-terminal sequences of E6 conserved only among high-risk HPV types are needed to direct p53 degradation. Most HPV-positive tumors have wild-type p53 whereas HPV-negative tumors hold mutant p53 (Crook et al. 1991a, Crook et al. 1992, Crook et al. 1991b, Schöndorf 2002, Wawroschek et al. 1999, Wieland and Pfister 1997).

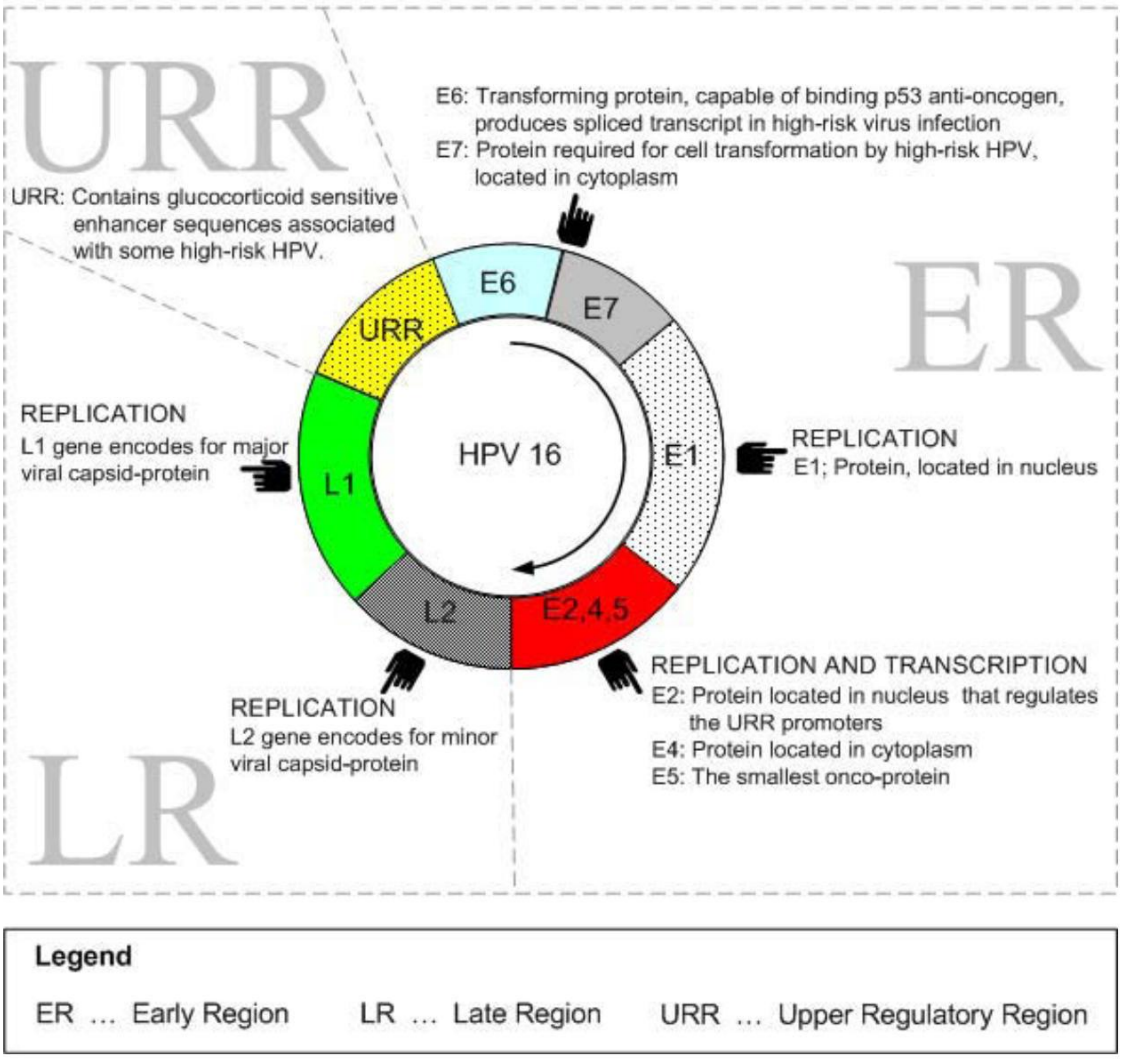



**Figure 6: Genomic organization of HPV type 16**

## 1.2 Prognosis of patients with cervical cancer

Cancer can begin in any organ or tissue of the body. The original tumor is called the primary cancer or primary tumor. Tumors that start from the primary cancer and spread to other organs are called metastases or micrometastases (AJCC 2002b). Cancer initially spreads to regional lymph nodes near the primary tumor. When cancer spreads to other organs or to lymph nodes far from the primary tumor, it is called secondary or distant tumor. Use of diagnostic methods such as immunohistochemistry (IHC) has enabled to differentiate primary from secondary tumors.

The prediction of how a patient's disease will progress, and whether there is a chance of recovery is called prognosis. To date, the cancer survival rate (the

percentage of surviving patients over a specific period of time) is important for making the prognosis of patients. Among other prognostic factors such as staging category, tumor grade, lymph-vascular space involvement (LVSI) and surgical margins, the LN status is the most important factor for the survival of patients with primary CCa. If LN metastases are present at the time of primary surgery (pN1) (Figure 8), the 5-year survival of women with CCa decreases from 85% to 50% (Delgado et al. 1990, Fuller et al. 1989). Prognosis of women with CCa is also influenced by estrogen and progesterone receptor levels in the tumor tissue, menopausal status, the general health of the patient, age, patient's attitude, effectiveness of treatment, cancer growth rate, etc (Yuan et al. 1999). Decisions for the appropriate therapy are taken after considering all possible prognostic factors.

To group patients with respect to prognosis, the International Federation of Gynecology and Obstetrics (FIGO), the American Joint Committee on Cancer (AJCC), and International Union Against Cancer (UICC) proposed the TNM and FIGO staging.

**TNM staging** T, N and M are mandatory parameters of the classification and stand respectively for the size or direct extent of the primary tumor (T – tumor), spread of the tumor to regional lymph nodes (N – node) and distant metastasis to other organs (M – metastasis) (UICC 2002).

**FIGO staging** FIGO is the acronym of the French name "Federation Internationale de Gynecologie et d'Obstetrique" or translated into English "International Federation of Gynecology and Obstetrics". FIGO staging represents the staging of CCa and is analogous to the TNM system. FIGO is a non-profit organization founded in 1954 to raise the standard of practice in obstetrics and gynecology and to promote the well-being of women and their children.

These systems describe the extent of cancer in the patient's body. Cancer staging is the most powerful predictor for disease survival; it is only determined at the time of primary diagnosis. Cancer staging takes into account several parameters of cancer progression and spread such as the size of the primary tumor, how deep it penetrated in the primary organ, if it goes through more than one organ, whether LN metastases are detected, whether metastases are found in secondary organs, etc (Figure 7). Moreover, treatment is often based on the

stage of the primary cancer. Staging systems are specific for each type of cancer. Sometimes more than one staging system can be used for the same type of cancer. Cervical cancer uses both TNM and FIGO staging systems that are similar to each other. Additionally, CIN grading system is used for the pre-malignant dysplastic changes.

Under FIGO stage I-IIA, the disease is relatively localized. It is noticed that although carcinoma is strictly confined in the cervix (FIGO I) or exceeds beyond uterus (FIGO II) and no lymph nodes are involved (pN0), the disease recurrence rate after treatment is still high. The cumulative 5-year survival of patients with CCa (FIGO: IA2, IB and IIA) is 92% (Eggen et al. 2007). A study of the International Federation of Gynecology and Obstetrics (FIGO) that evaluated the prognosis of patients with FIGO stage IA1 and IA2 resulted that 12 years after surgical treatment by conization, 0.3% of patients diagnosed with stage IA1 represented stage IIB (obvious parametrial involvement). Furthermore, 5.6% of patients with stage IA2 lesions developed invasive cancer and 3.4% died within the 5 years[4] after surgical treatment (Burghardt et al. 1991).

TNM staging is used for solid tumors and is divided in “clinical staging” and “pathologic staging”. It was developed to categorize the stage of breast cancer but is applicable for other cancer entities including the CCa as well (AJCC 2002a). Lymph node metastasizing remains the gold standard of staging; for this reason in Figure 8 we selected to illustrate the part of the TNM staging that demonstrates the classification of pathological lymph nodes (pN) in women with primary breast cancer (ACOG 2002, AJCC 2002b).

[4] 5 year survival rate is the percentage of patients who survive a certain time (at least 5 years) after a certain type of cancer (e.g. CCa) is diagnosed.

| | |
|---|---|
| **Stage 0** | Carcinoma in situ, cervical intraepithelial neoplasia Grade III. |
| **Stage I** | The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded). |
| | IA - Invasive carcinoma which can be diagnosed only by microscopy. All macroscopically visible lesions, even with superficial invasion, are allotted to Stage IB carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.0mm and a horizontal extension of not >7.0 mm. Depth of invasion should not be >5.0mm taken from the base of the epithelium of the original tissue, superficial or glandular. The involvement of vascular spaces, venous or lymphatic should not change the stage allotment.<br>IA1 - Measured stromal invasion of not >3.0mm in depth and extension of not >7.0 mm.<br>IA2 -Measured stromal invasion of >3.0mm and not >5.0mm with an extension of not >7.0mm. |
| | IB - Clinically visible lesions limited to the cervix uteri or preclinical cancers greater than Stage IA.<br>IB1 - Clinically visible lesions <4.0 cm.<br>IB2 - Clinically visible lesions >4.0 cm. |
| **Stage II** | Cervical carcinoma invades beyond uterus, but not to the pelvic wall or to the lower third of vagina. |
| | IIA - No obvious parametrial involvement.<br>IIB - Obvious parametrial involvement. |
| **Stage III** | The carcinoma has extended to the pelvic wall. On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. The tumor involves the lower third of the vagina. All cases with hydronephrosis or nonfunctioning kidney are included, unless they are known to be due to other cause |
| | IIIA - Tumor involves lower third of the vagina, with no extension to the pelvic wall.<br>IIIB - Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney. |
| **Stage IV** | The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV. |
| | IVA - Spread of the growth to adjacent organs.<br>IVB - Spread to distant organs. |

**Figure 7: Carcinoma of the cervix uteri: FIGO nomenclature (Quinn et al. 2006)**

In patients with microinvasive cervical cancers (FIGO IA1), lymphatic and hematologic metastases are rare but possible. Disease recurrence rate at this stage is less than 1% (Argenta et al. 2005). The incidence of pelvic lymph node metastases in patients with CCa (FIGO IIA and IB) ranges from 5% - 10% (Hacker 2000, Morrow and Curtin 1998). Approximately 12.5% of patients with early CCa (FIGO IB) and tumor positive LN (pN1) have disease recurrence and 9.4% die. Furthermore, 55% to 77% of pN1 patients with FIGO stage IIB have poor prognosis within 5 years period after treatment (Lee et al. 2006, Suprasert et al. 2005). The poor prognosis is significantly associated with the number of

micrometastases positive lymph nodes additionally to LN metastases (pN1) in patients with FIGO stage IIB CCa (Suprasert et al. 2005).

**pNX:** Regional lymph nodes cannot be assessed (e.g. previously removed, or not removed for pathologic study)

**pN0:** No regional lymph node metastases histologically, no additional examination for isolated tumor cells

- pN0(i-) No regional lymph node metastases histologically, negative IHC
- pN0(i+) No regional lymph node metastases histologically, positive IHC, no IHC cluster >0.2mm
- pN0(mol-) No regional lymph node metastases histologically, negative molecular findings (RT-PCR)
- pN0(mol+) No regional lymph node metastases histologically, positive molecular findings (RT-PCR)

**pN1:** Metastases in one to three axillary lymph nodes and/or in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent**

- pN1mi: Micrometastases (greater than 0.2mm, none greater than 2mm)
- pN1a: Metastases in one to three axillary lymph nodes
- pN1b: Metastases in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent**
- pN1c: Metastases in one to three axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent**.

**pN2:** Metastases in four to nine axillary lymph nodes, or in clinically apparent* internal mammary lymph nodes in the absence of axillary lymph node metastases to ipsilateral axillary lymph node(s) fixed to each other or to other structures

- pN2a: Metastases in four to nine axillary lymph nodes (at least one tumor deposit >2.0mm)
- pN2b: Metastases in clinically apparent* internal mammary lymph nodes in the absence of axillary lymph node metastases

**pN3:** Metastases in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent* ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes

- pN3a: Metastases in 10 or more axillary lymph nodes (at least one tumor deposit >2.0mm), or metastases to the infraclavicular lymph nodes
- pN3b: Metastases in clinically apparent* ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent**
- pN3c: Metastases in ipsilateral supraclavicular lymph nodes

**LEGEND**

pN … pathologic lymph node

* "Clinically apparent" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination.

** "Not clinically apparent" is defined as not detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination.

**Figure 8: Classification of pathologic LN according to TNM staging for Breast Cancer (AJCC 2002b)**

The association of disease prognosis and lymph node involvement in patients with primary CCa is now well established. Generally, if lymph nodes result to be

free of micrometastases or metastases (pN0), a good prognosis is expected. However, the clinical significance of nodal micrometastases remains controversial in several cancers (Nicastri et al. 2007). It is disappointing that although with pN0 status, disease recurrence still occurs. 10-15% of patients with CCa have disease recurrence after treatment even that their LN were pathologically free of micrometastases (pN0 status) (Delgado et al. 1990). Several studies discuss and different opinions exist about the potential prognostic role of undetected occult tumor cells or tumor cell clusters smaller than 0.2mm in LN (Kahn et al. 2006, Kurahara et al. 2007, Rena et al. 2007, Scheunemann et al. 2008).

Reports in the literature confirmed that in patients with small-size CCa (< 2.5cm, FIGO IA1), occult tumor cells can only rarely be detected by immunohistochemistry after a careful evaluation of sectioned lymph nodes and parametrical pelvic tissue. Disease recurrence in those patients could be due to occult residual tumor cells that were not removed by surgical treatment or that were disseminated during the surgical procedure and persisted in situ (Argenta et al. 2005, Horn et al. 2005). The 5-year disease free survival of patients with esophageal squamous cell cancer (Li et al. 2007) and the esophago-gastric cancer (MacGuill et al. 2007) significantly associates to the presence of occult tumor cells in pN0-classified LN. Present but neglected occult tumor cells or clusters in pN0 lymph nodes might be the explanation for the poor prognosis of patients with early CCa (will be also discussed in Paragraph 4.3.2). Consequently, consideration of small micro-metastases staging might be important for the future management of CCa.

### 1.2.1 Treatment of cervical cancer with regard to prognosis

Prognosis and treatment of cancer depends on its stage (Hall and Walton 1968). After treatment, 80 to 90% of women with FIGO I and 50 to 65% of women with FIGO II cervical cancer survive 5 years after diagnosis. Only 25 to 35% of women with FIGO III and 15% or fewer of those with FIGO IV CCa survive after 5 years. If only the surface of the cervix is involved, often the cancer is completely removed by removing a part of the cervix by using the loop electrosurgical excision procedure, a laser, or a cold knife (Steed et al. 2006). Cryotherapy may be also used to destroy the cancer by freezing it. These treatments preserve a woman's ability to have children. Because cancer can recur, women are advised

to return for examinations and Pap tests every 3 months for the first year and every 6 months after that (Cox 2003). If cancer has begun to spread in the pelvic area, hysterectomy plus removal of surrounding tissues, ligaments, and lymph nodes (radical hysterectomy) is necessary. The ovaries may be also removed. Normal functioning ovaries of young women are not removed. Alternatively, radiation and/or chemotherapy therapy may be used. Radiation therapy alone is ineffective in about 40% of women with large or extensive cancers (Cannistra and Niloff 1996). When the cancer has spread extensively or recurs, chemotherapy is usually used. However, chemotherapy reduces the cancer's size and controls its spread in only 25 to 30% of women treated, and this effect is usually temporary (Figure 9) (Cannistra and Niloff 1996, Schneider and Hertel 2004).

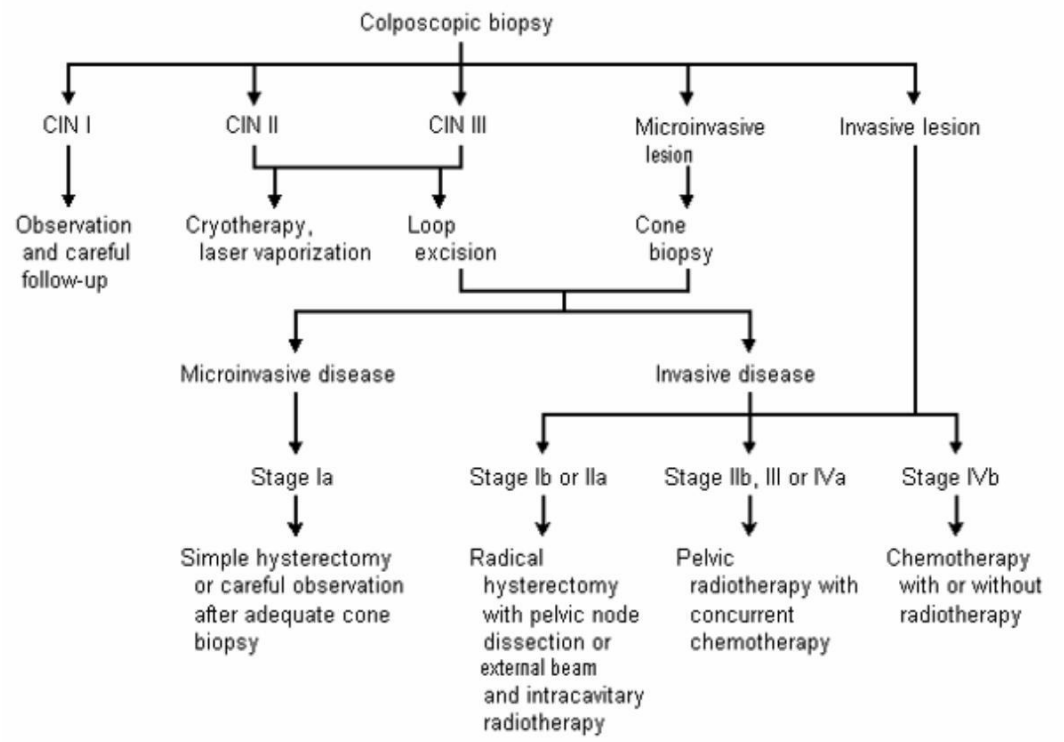


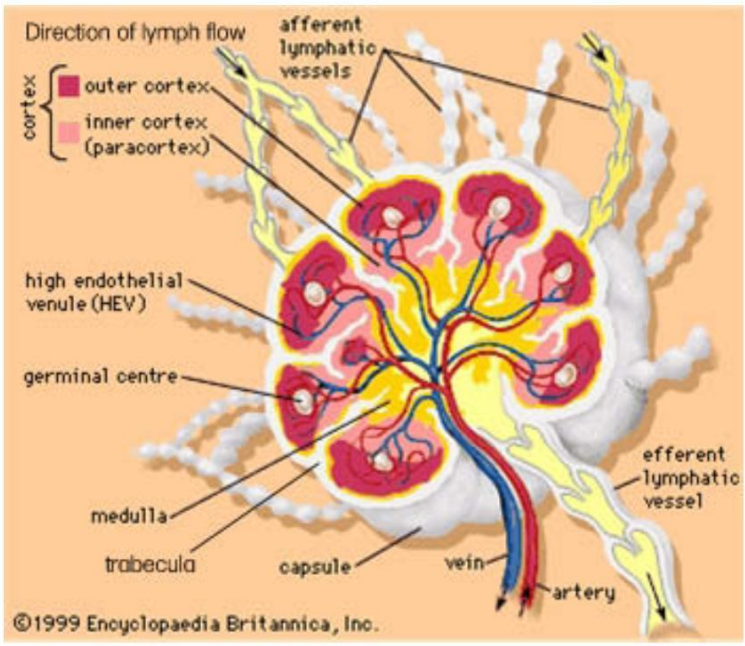

**Figure 9: Algorithm for the management of Papanicolaou smear findings and invasive CCa (Cannistra and Niloff 1996)**

### 1.2.2 The anatomy of a lymph node

The lymphatic system is a major component of the immune system. It is a complex network of lymphoid organs that produce and transport lymph fluid from tissues to the circulatory system. The lymphatic system consists of complex capillary networks, lymphatic vessels, lymph trunks and ducts, lymph nodes and extra nodal (lymphoid) tissues. The system has three interconnected functions: It

collects the excess fluids and proteins from body tissues and carries them back to the bloodstream, transports fat and chyle to the circulatory system, absorbs fatty acids and, furthermore, produces immune cells such as lymphocytes, monocytes and plasma cells (antibody producing cells). When blood plasma pours out of the capillaries of the circulatory system and becomes interstitial fluid, it fills the space between individual cells of tissue. Because of the hydrostatic pressure, plasma is filtered out of the capillaries and due to the oncotic pressure it is absorbed back in the capillaries. Outside the capillaries, the plasmatic fluid mixes with the interstitial fluid. Consequently, the interstitial volume increases gradually. The oncotic pressure returns the fluid to the capillaries. After entering the lymphatic system the fluid is called lymph; it has almost the same composition as the original interstitial fluid. The excess interstitial fluid which is collected by the lymphatic system is then processed by lymph nodes acting as filters.

Human body has about 500-600 lymph nodes (Figure 10). They contain an internal honeycomb of reticular connective tissue filled with lymphocytes that collect and destroy bacteria and viruses. Here, foreign antigens can be trapped and exposed to cells of the immune system for destruction. The central role played by lymph nodes in filtering microorganisms and other undesired substances from the blood is important for the functioning of the immune system but also makes lymph nodes vulnerable to cancer. The lymphatic spread of carcinoma is primarily embolic. As cancerous cells escape from the primary tumor, they penetrate into lymphatic vessels and can become trapped and concentrated in lymph nodes, where they proliferate. The involved nodes prevent further spread until the node is completely overwhelmed by carcinoma. Further embolic spread of cancer is through the collateral channels. Each new node that is involved tends to make the spread of new embolus more difficult. Spread from one node to another does not seem to be common. Almost all cancers have the potential of spreading to lymph nodes, a condition that greatly complicates treatment (Encyclopaedia Britannica 2010, Gilchrist 1940). The migrating cancer cells, after entering the lymphatic and blood vessels, circulate through the bloodstream and metastasize in a distant organ of the body.




Each lymph node is surrounded by a capsule that is an outer layer of connective tissue. The capsule extends inside the LN to form trabecula. Thin reticular fibers form a supporting meshwork inside the node. The concave side of the lymph node is called “hilus”. The artery and vein are connected at the hilus and respectively allow blood to enter and leave the LN. Underlying the capsule is the cortex, a region containing basically inactivated B and T lymphocytes as well as numerous accessory cells such as dendrite cells and macrophages. The cortex is further divided into the outer and inner cortex (paracortex). Lymph and its associated cells enter the LN through the efferent lymphatic vessels. These vessels may drain directly from the lymphatic capillaries or may be connected to a previous node. Lymphocytes generally enter the LN through specialized blood vessels that are called high endothelial venules (HEV). HEV contain a single layer of large endothelial cells that possess surface receptors specific for B and T lymphocytes. As large endothelial cells pass through HEV, they bind to the receptors and are carried into the inner cortex of the LN. Most of the lymphocytes within a LN have yet to encounter an antigen; therefore, they must migrate to regions where they will be most effective in recognizing foreign agents. B lymphocytes then migrate to the outer cortex and join specialized dendrite cells and macrophages to form follicles. After encountering a foreign antigen, the B lymphocytes get activated and are surrounded by a more tightly packed association of dendrite cells and macrophages, forming a germinal centre. The germinal centre together with its mantle (a ring zone of resting B cells and dendritic cells) compose a secondary follicle, which is the site of antigen-dependent B-lymphocytes maturation. Then the activated B lymphocytes migrate through the inner cortex to the medulla, where they proliferate as antibody-secreting plasma cells. T cells that entered the LN through HEV remain in the inner cortex. There, the cortical macrophages and dendritic cells present antigenic peptides to the T cells that did not encounter antigen yet, stimulating them to activate helper T cells or cytotoxic T lymphocytes. All activated lymphocytes migrate through the medulla and enter the lymphatic circulation through the efferent lymphatic vessel, which drains either nearby LN or ultimately into the thoracic duct, a major vessel of the lymphatic system (Encyclopaedia Britannica 2010, Gilchrist 1940).

**Figure 10: Anatomical structure of a lymph node (Encyclopaedia Britannica 2010)**

While normal human lymph nodes range in size from a few millimeters to 1-2cm, malignant lymph nodes tend to be bigger than normal. However, the size of the lymph nodes cannot be used as a sole criterion for the differential diagnoses since nodes may also be enlarged due to infection that results in enhanced proliferation of activated T and B cells. In some cases, they may be enlarged due to past infections (Encyclopaedia Britannica 2010). When the body is fighting an infection, lymphocytes multiply rapidly and produce a characteristic swelling of the lymph nodes. Moreover, not only enlarged lymph nodes may contain metastases. Metastatic deposits can be also found in small nodes that macroscopically look healthy. In the routine, the metastasis or micrometastasis positive nodes are diagnosed by the conventional histology.

### 1.2.3 Diagnostic role of tumor markers

Tumor markers are measurable biochemical indicators, selectively produced by the tumor or non-tumor cells as a response to the presence of a tumor. They are tumor-associated antigens that can be detected in blood and other tissues (Benjamin 1995, Hussain et al. 2010). Generally, an elevated level of a tumor marker can indicate cancer; however, other causes of elevation are possible. For this reason, tumor markers can be classified in two groups: Cancer-specific markers and tissue-specific markers. Tumor markers are used in oncology for the following purposes:

- to early detect and diagnose the presence of a tumor,
- to monitor the disease progress or recurrence and patient's prognosis,
- to determine the differential diagnosis,
- to screen a healthy or high-risk population for the presence of cancer and
- to evaluate the response to treatment response in case of malignancy.

They can be found in high quantities in blood, urine, or body tissues of patients with certain types of cancer. Tumor markers can be either tumor-derived (produced by the tumor cells) or tumor-associated (produced by the human body) in response to the tumor cells. They can be cell surface antigens, cytoplasm proteins, enzymes or hormones. As a consequence of the discovery of

monoclonal antibodies, a range of tumor markers has been discovered to date. Currently, tumor markers are basically used to assess the tumor response to treatment and to diagnose the disease recurrence. In this study, we used four tumor markers to detect the presence of micrometastases and occult tumor cells in LN tissue of patients with CCa. We tested whether the following four tumor markers are specific for LN micrometastases:

- $p16^{INK4a}$ which is a surrogate marker for high risk HPV
- Cytokeratin markers CK19 and AE1/3 which are epithelial cell markers
- Viral oncogene transcripts (HPV mRNA) which are constitutively expressed in all CCa cells

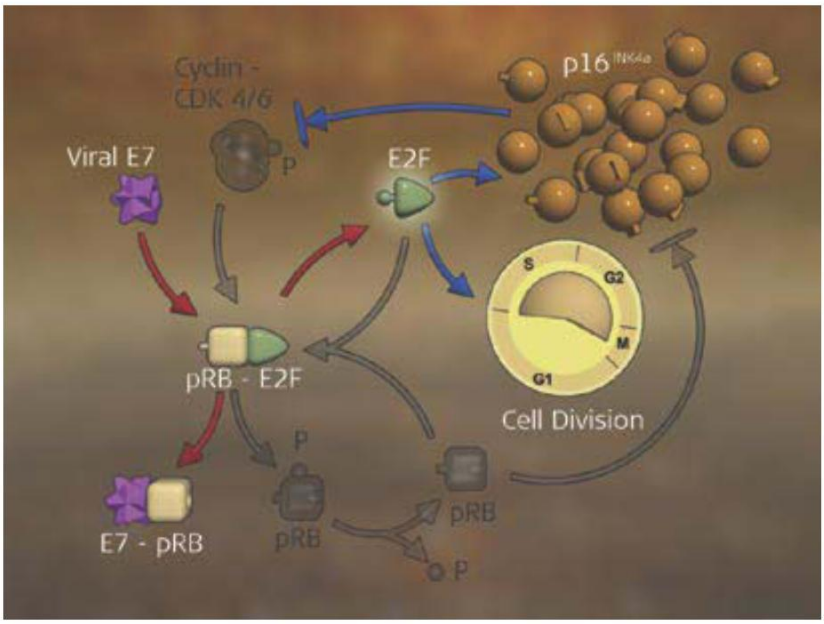
All these markers have been used by different studies but their reliability for the detection of LN micrometastases was not researched yet.

#### 1.2.3.1 $p16^{INK4a}$: A surrogate marker of HR-HPV

$p16^{INK4a}$ is a member of the INK4a family of cyclin-dependent kinase inhibitors and is known as a negative regulator of cell cycle progression and differentiation. An enhanced expression of the $p16^{INK4a}$ protein might represent a useful biomarker for cells with activated expression of HPV oncogenes (zur Hausen 2000). The evolution of cell cycle is coordinated through complex mechanisms that manage the expression and post-translational modification (e.g. phosphorylation) of cell-cycle regulating proteins. Among these proteins, the cyclin-dependent kinase inhibitor $p16^{INK4a}$ is a tightly regulated protein involved in the cell cycle control. It plays a pivot role in the retinoblastoma protein (pRB) mediated control of the G1-S- phase transition of the cell cycle. Expression of the HR-HPV oncogenes E6 and E7 is required to initiate and maintain the transformed phenotype of epithelial cells in pathogenesis of cervical cancer and its precursors. Expression of the HR-HPV E7 gene in replicating epithelial cells results in disruption of the pRB-E2F complex and functional inactivation of pRB. This leads to strong overexpression of the $p16^{INK4a}$ independent from the HR-HPV type. The binding of E7 oncoprotein leads to inactivation of the normal pRB pathway regulation. When the E7 oncoprotein binds to pRB, the transcription factor E2F is released and thereby allows the cell to proceed through the $G_1$/S

transition. The negative feedback control from the expressed $p16^{INK4a}$ to CDK4/6 has therefore no effect (Figure 11) (Biolegend 2009, DakoCytomation 2010, Schöndorf 2002).

A strong overexpression of $p16^{INK4a}$ protein has been reported for the cervical cells that are transformed by high-risk HPV (DakoCytomation 2010, Schöndorf 2002, Zuna et al. 2004). HPV-16 E6 and E7 oncoproteins are able to abrogate negative growth regulatory signaling pathways of the host cell through interaction with p53 and pRB tumor suppressor proteins. Consequently, after transformation develops, the proliferation of HR-HPV infected cells becomes de-regulated. Activation of the E6 and E7 tumor proteins of HR-HPV types in the respective epithelial cells is the decisive element of degeneration. The genetic instability of the tumor protein HR-HPV E7 is one of the main reasons of cancer (DakoCytomation 2010).




**Legend**:

- pRB = retinoblastoma protein
- pRB-P = phosphorylated retinoblastoma protein
- E7 = oncoprotein from oncogene E7
- E2F = transcription factor
- CDK4/6 = cyclin-dependent kinase 4 and cyclin-dependent kinase 6
- $p16^{INK4a}$ = proposed cell cycle regulator

**Figure 11: The mechanism leading to overexpression of $p16^{INK4a}$ in the HPV-transformed cell (DakoCytomation 2005)**

Antibodies against cyclin-dependent kinase inhibitor $p16^{INK4A}$ protein, allow staining of tissue sections of the HPV associated cervical cancer. As $p16^{INK4A}$ is overexpressed in cell nucleus and cytoplasm, the whole cell is stained in brown color. It clearly detects the tumor cells of the primary CCa. Nowadays $p16^{INK4a}$ is used as a marker for CCa; but, it could also be a good marker for detecting all lesions associated with the papillomavirus and might, therefore, contribute to diagnose the cervical dysplasia and reactive lesions (Miller 2002). An overexpression of this protein in distant micrometastases and malignant or transforming cells of LN of patients with HPV16 positive CCa could suggest $p16^{INK4a}$ as a potential marker for LN as well.

#### 1.2.3.2 Cytokeratins as markers

The cytoskeleton of almost all eukaryotic cells is build up jointly of microtubules and microfilaments where cytokeratins (CK) are intermediate filaments. The CK family is a highly complex water-soluble multi-gene group of polypeptides, the molecular weight of which varies from 40 to 68 kDa. 20 distinct CK are known today, not including the so-called trichocytic that are keratins present only in hair and nail-forming epithelia. The classification and numbering of cytokeratins (CK1-CK20) is based on the catalogue of Moll et al. (Moll et al. 1982). They can be divided into an acidic (type I) and a neutral-basic (type II) subfamily. CK expression in the uterine cervix is complex. Cytokeratin antibodies with broad specificity (pan CK antibodies such as AE1/3, KL1, MNF116 and CK11), are used as markers to distinguish carcinomas from the majority of non-epithelial malignant tumors. Endocervical epithelium contains simple epithelial-type keratins 8, 18 and 19 while ectocervical epithelium contains stratification-type keratins 5 and 7 as well as several other cytokeratin peptides (Moll et al. 1982). Of the simple cytokeratin polypeptides, cytokeratins 8, 18 and 19 are expressed in most of the cells of carcinomas (Benjamin 1995, Moll 1994). Furthermore, the lymph node tissue does not contain cytokeratins which means that CK could serve as potential markers for LN metastasis originating from epithelial tumors. Carcinomas generally express CK prototypes that at least partially represent the pattern of the putative cells of origin. The consequent profile reflecting both epithelial type and differentiation status might therefore be useful for tumor

diagnosis. Adenocarcinomas generally express the simple epithelial-type CK8, CK18, CK19 and, frequently, CK7. However, some types of carcinomas may deviate from the patterns of their normal counterpart. Certain carcinomas may lose the ability to express one or more CK found in the putative tissue of origin.

#### 1.2.3.2.1 Cytokeratin 19: A marker of epithelial cells

Cytokeratin 19 (CK19) belongs to the family of 20 related polypeptides that are constituents of the intermediate filaments of epithelial cells. It is the lowest molecular-weight keratin (40-44 kD). CK19 is a heterotetramer composed of two type I and two type II keratin subunits. Unlike other cytokeratins, CK19 lacks a C-terminal non-helical extension. Low molecular weight cytokeratins have been assessed as markers of potential invasion in cervical intraepithelial neoplasia (Benjamin 1995). As shown in Table 2, among others, cytokeratin 19 is expressed in higher amounts in the transitional epithelia that consist of superficial basal and intermediate cells as well as luminal, basal and myoepithelial cells in a few complex epithelia. CK19 is normally not expressed in stratified squamous epithelia, but may be present in modified squamous epithelium invaded by lymphocytes as well as in basal cells in non-keratinizing stratified squamous epithelium (Dako 2010a). Cytokeratin 19 can be up-regulated by vitamin A and is thought to play a critical role in embryogenesis. It interacts with the pinin protein and is shown to be modified by phosphorylation (Ser10, Ser35). The A53-B/A2 monoclonal antibody recognizes human cytokeratin 19 and is useful for Western blotting. This antibody is also reported to be useful for immunoprecipitation, immunohistochemistry (paraffin sections), and ELISA (Biolegend 2009). Furthermore, CK19 is proposed to be a potential marker for identifying lymphovascular invasion with much greater sensitivity than the H-E (Hematoxylin-Eosin) staining (Alexander-sefre et al. 2002).

**Table 2: CK19 expression according to the Moll catalogue (Moll et al. 1982)**

| Cell types | Acidic type | CK 19 |
|---|---|---|
| Stratified squamous epithelia | - Basal cells | ++[a] |
| Transitional epithelium | - Superficial cells | +++ |
| | - Basal and intermediate cells | +++ |
| Complex epithelia | - Basal and myoepithelial cells | ++ |
| | - Luminal cells | +++ |
| Simple epithelia | - Biliary and pancreatic ducts, lung alveoli, endometrium, renal collecting ducts | +++ |
| | - Stomach (foveolar epithelium), small and large intestine | +++ |
| | - Endocrine glands, neuroendocrine cells | +++[b] |
| Non-epithelial cells | - Some smooth muscle cells, myofibroblasts, arachnoid, some endothelial cells | + |

**Legend**

+++ Widespread occurrence, large amounts of CK19

++ Varying distribution, moderate amounts of CK19

\+ Limited occurrence, sparse amounts of CK19

[a] Basal cells in mucosa but not in skin.

[b] Some endocrine cells, particularly Merkel cells of skin

#### 1.2.3.2.2 Cytokeratin AE1/3: A cocktail marker of epithelial cells

AE1/3 is a cocktail epithelial marker of combined high and low molecular weight keratin AE1 and AE3. This mixture of antibodies reacts with almost all epithelia and does not show cross-reactivity with other intermediate filaments. Thus, it is considered to be a good marker for differentiating epithelial cancer from the non-epithelial one. Existing studies find AE1/3 as a highly sensitive marker in delineating sentinel lymph node metastasis, not only cell clusters but also isolated cancerous cells (Moll 1994, Moll et al. 1982). AE1/3 is considered to be a better marker than other pan CK or Cam 5.2 for the detection of lymph node metastases (Lerwill 2004). However, the value of AE1/3 immunohistochemistry on frozen sections is recommended to be studied further (Lerwill 2004).

#### 1.2.3.3 Viral oncogene transcripts (HPV mRNA)

The HPV oncogene transcripts represent an ideal marker for LN of patients with CCa. They have the highest potential for accurate identification of viable tumor cells compared to established markers such as the cytokeratins (Häfner et al. 2007, Van Trappen et al. 2001). PCR technology is the backbone of molecular biological research. RT-PCR is a highly sensitive method for detecting rare tumor cell-derived mRNA, allowing the diagnosing of tumor dissemination in early stages (Häfner et al. 2007, Max et al. 2002). HPV mRNA can be detected with high sensitivity in lymph nodes; one tumor cell can be detected in a background of $10^6$-$10^7$ other cells. Consequently, it is a prerequisite for tumor progression and required for the maintenance of the transformed phenotype (Häfner et al. 2007).

## 1.3 Aims of the study

This study aims to validate four potential markers for lymph nodes of women with primary cervical cancer associated with HPV infection (HPV16). First, the four markers ($p16^{INK4a}$, CK19, AE1/3 and HPV mRNA) are tested for their reliability to detect micrometastases and disseminated or sporadic tumor cells and tumor cell clusters in the LN of patients with CCa. $P16^{INK4a}$ shows great promise as a marker of lesions associated with high-risk HPV but it is still questionable whether $p16^{INK4a}$ might serve as a predictor of the LN status of patients with primary CCa. Additionally to $p16^{INK4a}$ we have chosen to evaluate the reliability on the epithelial markers AE1/3 and CK19 that could also be used to diagnose distant tumors in lymph nodes of patients with primary epithelial CCa. Apart of the IHC markers, a molecular marker at the RNA level (HPV mRNA) is likely to be a valid marker for LN as well. To test the reliability on these selected markers, in the coming chapters we will:

- validate the staining quality of the selected IHC markers ($p16^{INK4a}$, CK19, AE1/3)
- determine the statistical agreement between the three IHC markers
- determine the statistical agreement between each of the three IHC markers and the molecular marker HPV mRNA

- look at the limitations of this study and discuss about the reliability of the selected markers for forthcoming studies
- discuss the necessity to evaluate the prognostic role of the occult tumor cells and clusters smaller than micrometastases (<0.2mm) in LN of patients with CCa and accordingly the prospective view of CCa management schemas.

# Chapter 2: Patients, materials and methods

48 women diagnosed with cervical cancer (FIGO stage IA1-IIB) and positive for HPV16 were enrolled in this study. These women were included in a prospective multi-center study that intended the evaluation of the "sentinel lymph node concept" (SLN concept) for patients with primary cervical cancer (Altgassen et al. 2002, Altgassen et al. 2008). The study is managed by the Clinic of Obstetrics and Gynecology of the University Clinic Jena, Germany. All enrolled women underwent radical hysterectomy and complete lymphadenectomy. 120 sentinel lymph nodes were evaluated in this study. The identification of SLN is done with the help of a radioactively labeled protein, a marker substance (Patent-Blue), or both as described in Paragraph 2.2. The sentinel lymph nodes were removed and evaluated for metastases, micrometastases, and occult tumor cells. The evaluation was performed at the Institute of Pathology and Clinic of Gynecology of the University Clinic Jena by the use of the immunohistochemical approach (IHC) (Paragraph 2.3.1) and real-time reverse transcription PCR technology (RT-PCR) (Section 2.3.2).

A total of 120 SLN were assessed for metastatic cells by using the IHC biomarkers $p16^{INK4a}$, CK 19, AE 1/3 and the molecular marker HPV-mRNA. All these lymph nodes were initially evaluated by conventional histopathological examination with the result that 35 SLN were positive and 85 were negative for micrometastases or metastases.

We set up an access database to store the data (Paragraph 2.4) that is subsequently statistically analyzed. Descriptive statistics is used to measure the

validity of the potential diagnostic makers for lymph nodes of CCa patients. The corresponding statistical methods are explained in the Paragraph 2.5.

## 2.1 Patients included in the study

All 48 enrolled women with primary CCa and were treated and followed up at the Clinic of Gynecology of the University Clinic Jena. The mean age of the patients or the arithmetic value of the central tendency (calculated by adding up all terms and then dividing by the number of terms of distribution) was 44, ranging from 27 to 75. The cervical cancer was verified by conventional histology in all 48 women. 44 of them (91.67%) were diagnosed with squamous cell carcinoma and 4 (8.33%) with adenocarcinoma. According to FIGO-staging (explained in Paragraph 1.2), all 48 women had tumor stage I or II (Figure 12).

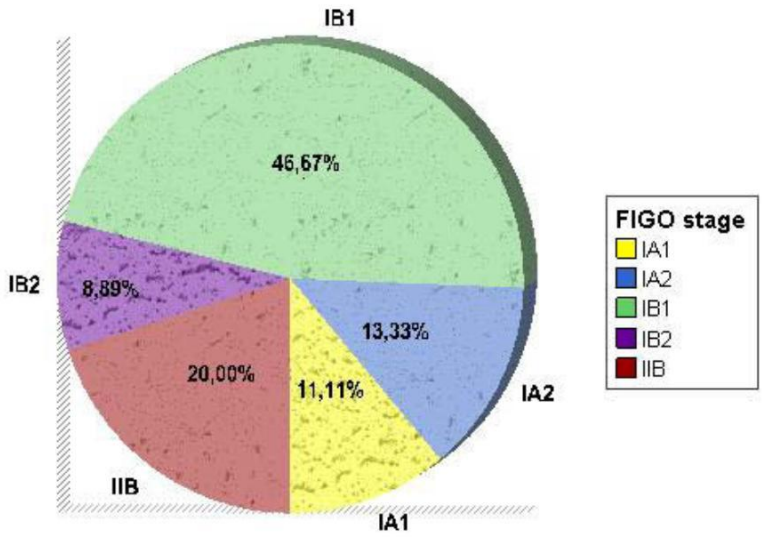



**Figure 12: FIGO status of the patients enrolled in the study**

After approval from the ethical committee of the University Clinic Jena, a written consent-form was signed by each woman before participating in the study. Pregnant women, women that could not undergo surgery as well as the ones who had a previously pelvic or paraaortic lymphadenectomy were excluded from the study. Furthermore, not eligible to participate in the study were also women with pre-operative diagnosis of nodal metastasis, previous pelvic or paraaortic lymphadenectomy, lymphoscintigraphy within 14 days prior to surgery, verified

secondary tumor of the adnexes, and women with known allergy from labeling substances (Altgassen et al. 2002, Altgassen et al. 2008).

## 2.2 Detection of lymph nodes

All lymph nodes were identified and collected for the prospective multi-centre study aiming to evaluate the SLN concept in patients with CCa. Necessary data corresponding to the SLN identification was stored in the project database and managed at the Clinic of Obstetrics and Gynecology, University Clinic Jena (Altgassen et al. 2002, Altgassen et al. 2008).

The SLN identification was attained by injecting either a radioactively labeled protein (the albumin: Technetium$^{99m}$-Albu-Res®/Nanocoll®) or a marker substance (Blue Dye®). These marker substances are routinely used to label the lymphatic drainage. The introduction of the blue dye mapping by Morton and colleagues was the first step that led towards determining the importance of the SLN biopsy (Morton et al. 1989). The substance was injected subepithelially at the location of the primary tumor of patients with melanoma. Then, an incision was done over the expected location of lymphatic drainage. The lymphatic drainage was clearly stained and identified. This technique of the intraoperative lymphatic mapping was first presented as poster at the World Health Organizations´ II$^{nd}$ International Conference on Melanoma, followed by a written report (Morton et al. 2003, Morton et al. 1992). However, this way of LN detection is not associated with the tumor size, nodal status and FIGO stage. Seldom anaphylactic reactions are possible, therefore, before using the staining substances Albu-Res®/Nanocoll® or Blue Dye® was confirmed that the women never had hypersensitivity from exposure to allergens that marker substances contain. For example, Blue Dye® is often used in food production, therefore, before injection, it is confirmed that none of the women ever had allergies from food. Prior to using the staining substances, it was also ensured that the patients had to undergo a surgical treatment. Conclusively, both the patient and anesthesiologist agreed to use the labeling substances (Altgassen et al. 2006).

The day prior to surgery, 60 MBq radioactively labeled albumin Technetium$^{99m}$ bound to 1 ml Albu-Res® or Nanocoll® was injected subepithelially around the

tumor into the four quadrants of cervix by using an insulin syringe (Figure 13). The injection dose of 0.25 ml was given at an interval of 3 hours: 3, 6, 9 and 12 o'clock position of the cervix. The latest injection did not exceed 20 hours from the time of surgery and was performed slowly to allow fluid transmit and avoid backflow through the injection canal.

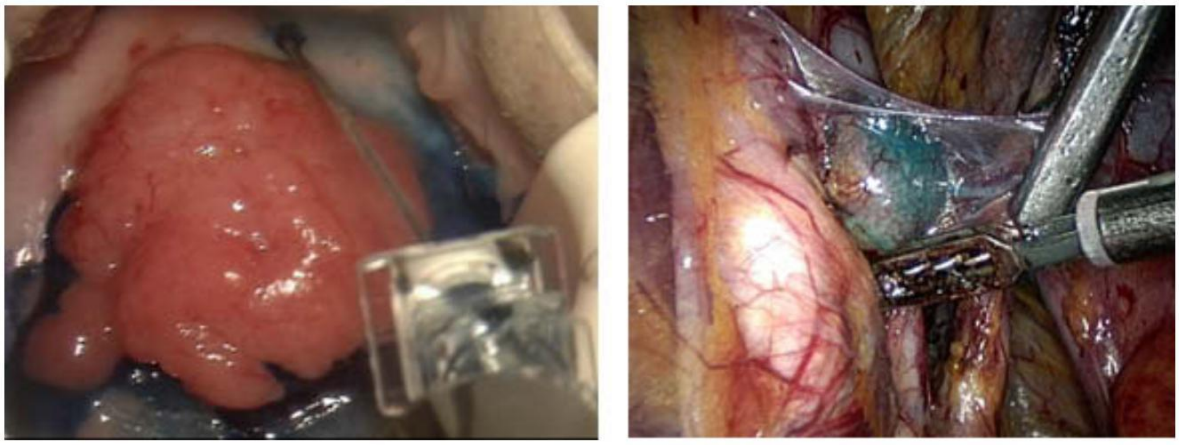


**Figure 13: Sentinel lymph node detection by injecting labeling substances via lymphatic channels (Altgassen et al. 2006, Schneider 2007)**

Additionally or alternatively, 4ml Patent Blue Dye® was injected subepithelially in the four quadrants of cervix at the day of surgery by using a 5 ml spinal syringe. The injection dose (0.5 ml) was given at an interval of 3 hours: 3, 6, 9 and 12 o'clock position of the cervix. The last injection was done directly after the general anesthesia and prior to surgery. It was important not to apply the injections directly into the tumor and to avoid backflow at the cervical canal. 4 ml Patent Blue Dye® was used as doses with lower quantity than 4ml injected Blue Dye® show a high failure rate (Altgassen et al. 2006, Schneider 2007). If a combined labeling procedure was chosen, radioactively labeled albumin was injected the afternoon before the day of surgery and Blue Dye® was injected directly before surgery, after induction of general anesthesia. The radioactive labeled SLN were localized with the help of a γ detector probe.

According to Altgassen et al. (Altgassen et al. 2002, Altgassen et al. 2008, Altgassen et al. 2006), the combination of radioactively labeled albumin Technetium$^{99m}$ and Blue Dye® gives a higher Detection Rate (93.5%) and higher sensitivity (80.3%) in comparison to usage of only one marking substance alone. However, the best negative predictive value (NPV) was after Technetium$^{99m}$ was

used alone (Table 3). In this study, that is part of the cohort study Uterus III (Altgassen et al. 2002, Altgassen et al. 2008) only 4.2% of the SLN (5/120) from 2 women were identified by using Technetium$^{99m}$ alone. For the staining of the rest of 95.8% SLN (115/120) both Technetium$^{99m}$ and Blue Dye® were used.

**Table 3: Validity of "sentinel" concept in patients with CCa (data taken from (Altgassen et al. 2008))**

| Validity parameter | Technetium$^{99m}$ | Patent Blue Dye® | Technetium$^{99m}$ and Patent Blue Dye® | Total |
|---|---|---|---|---|
| **Detection Rate**[1] (95%CI) | 81.8% (69.0% to 91.0%) N[4]=45 patients | 82.0% (75.9% to 87.2%) N[4]=160 patients | 93.5% (90.3% to 96.0%) N[4]=318 patients | 88.6% (85.8% to 91.1%) N[4]=523 patients |
| **Sensitivity**[2] (True positive: cp/fn) (95%CI) | 71.4% (29.0% to 96.4%) (5/7) N[4]=45 patients | 72.7% (54.4% to 86.7%) (24/33) N[4]=157 patients | 80.3% (68.6% to 89.1%) (53/66) N[4]=302 patients | 77.4% (68.2% to 85.0%) (82/106) N[4]=504 (3 patients were excluded) |
| **NPV**[3] (True negative: cn/fn) (95%CI) | 95.0% (83.0% to 99.4%) (38/40) N[4]=45 patients | 93.2% (87.5% to 96.8%) (124/133) N[4]=157 patients | 94.8% (91.2% to 97.1%) (236/249) N[4]=302 patients | 94.3% (398/422) N[4]= 504 patients |

[1] *Detection Rate* is the percentage of patients with a SLN identified during surgery according to the labelling substance divided by total number of patients in the study.
[2] *Sensitivity* is the percentage of patients with SLN metastasis in comparison to the total number of patients with nodal metastasis.
[3] *Negative Predictive Value* is the probability by which no lymph node metastases are found in negative SLN.
[4] Number of patients with SLN detection

Generally, a primary surgical approach with radical hysterectomy and pelvic adenectomy is performed starting from the early FIGO stages (IA, IB) (Paragraph 1.2.1). Complete lymph node removal is frequently associated with significant complications. According to the "SLN concept", if 100% of positive SLN are stained (sensitivity: 100%), this concept would be valid for the CCa as well; consequently, only selective SLN would be removed through laparoscopic approach (Dargent et al. 2000). Therefore, the "SLN concept" would be very important for the management of early invasive cancer. The Uterus III study (Altgassen et al. 2002, Altgassen et al. 2008) accepted the sensitivity of 90% as the minimal limit, but it was shown that the best overall sensitivity was only 80.3% which suggests that the SLN concept is not satisfactory for the cervical cancer.

### 2.2.1 Lymphadenectomy

Tumor cells tend to spread mostly in the lymphatic region around the vessels of the pelvic wall. Actually, they spread to the iliac lymph nodes higher in the pelvis, the aortic lymph nodes, and the nodes above the collarbone and occasionally to the groin nodes (Figure 14). All women enrolled in this study underwent complete lymphadenectomy and radical hysterectomy. The paraaortic and regional lymph nodes were completely removed. Laparoscopic surgery, also called minimally invasive surgery (MIS), is a reliable surgical method for lymphadenectomy or radical hysterectomy in which operations in the abdomen are performed through small incisions (Barranger et al. 2003). The surgical lymphadenectomy and hysterectomy can also be done through an open procedure. Comparing to an open procedure, laparoscopic surgery offers a number of advantages to the patient. Some of these advantages are:

- reduced blood loss, consequently less risk of needing a blood transfusion,
- smaller incision, consequently less pain and shorter recovery time,
- less pain, consequently less pain medication is needed,
- shorter hospital stay and faster recovery,
- reduced exposure of internal organs to possible external contaminants and, consequently, reduced risk of acquiring infections.

Due to certain reasons, our patients underwent the complete pelvic lymphadenectomy either by laparoscopy or by an open approach.

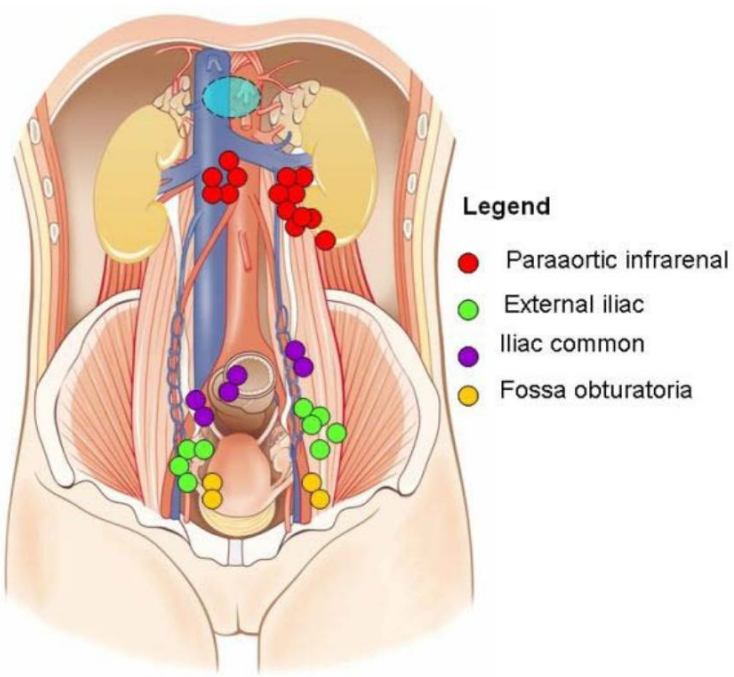



**Figure 14: Tomography of lymph nodes frequently trapping tumor cells from CCa**

## 2.3 Methods used for detection of micrometastases in SLN

Principally, there are two methods to identify tumor presence in lymph nodes:

- By the direct use of IHC methods starting with a preparation of sections from selected lymph nodes. The sections are then stained with the help of antibodies against tumor markers
- By indirectly evaluating the specific marker-gene transcripts such as viral mRNA in the lymph nodes. This is achieved by the use of RT-PCR molecular biologic method (nested or real-time PCR).

As shown in Figure 15, we divided each SLN tissue into two parts. A section of 1-2mm thickness was immediately snap-frozen in liquid nitrogen and stored at -80$^{o}$ C until the RNA extraction. The rest of the SLN tissue was processed for routine histopathological diagnosis. The paraffin embedded material was available for IHC examination by conventional histology and the selected markers performed in this study.

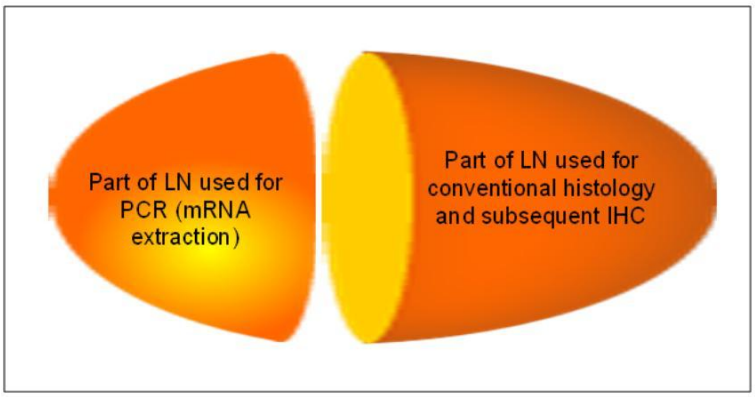



**Figure 15: LN used for routine histology, IHC, and molecular biologic evaluation**

In the following Paragraphs 2.3.1 and 2.3.2 the procedures of immunohistochemistry and PCR performed by using the four potential tumor markers for lymph nodes ($p16^{INK4a}$, CK19, AE 1/3, and HPV mRNA) will be briefly explained.

### 2.3.1 Immunohistochemistry

Immunohistochemistry is an important diagnostic method. Its name comes from the latine words “immuno” and “histo”. "Immuno“ refers to antibodies used in the IHC procedure and "histo" denotes the tissue. The principle of IHC is to detect antigens in tissues by use of antibodies. It gives a supreme amount of information about antigen detection in pathological tissues. The antibody to a specific antigen is labeled by a signal molecule that is an enzyme peroxidase or alkaline phosphatase (Figure 16). The signal molecule together with the specific antibody substrate is called “biomarker” due to the fact that it produces a colored and insoluble product via which the antigens are marked (Benjamin 1995). In our study, antigens are the dysplastic cells or cell-clusters located in lymph nodes. With the help of biomarkers ($p16^{INK4a}$, CK19, AE1/3) staining of the antigens (tumor cells) located in the lymph nodes of patients with cervical cancer is performed. Formalin-fixed paraffin-embedded tissue sections from 120 LN were stained immunohistochemically using antibodies against cytokeratins and $p16^{INK4a}$. To confirm the diagnosis a section of each LN was stained with hematoxylin and eosin.

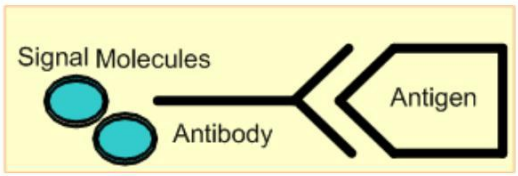
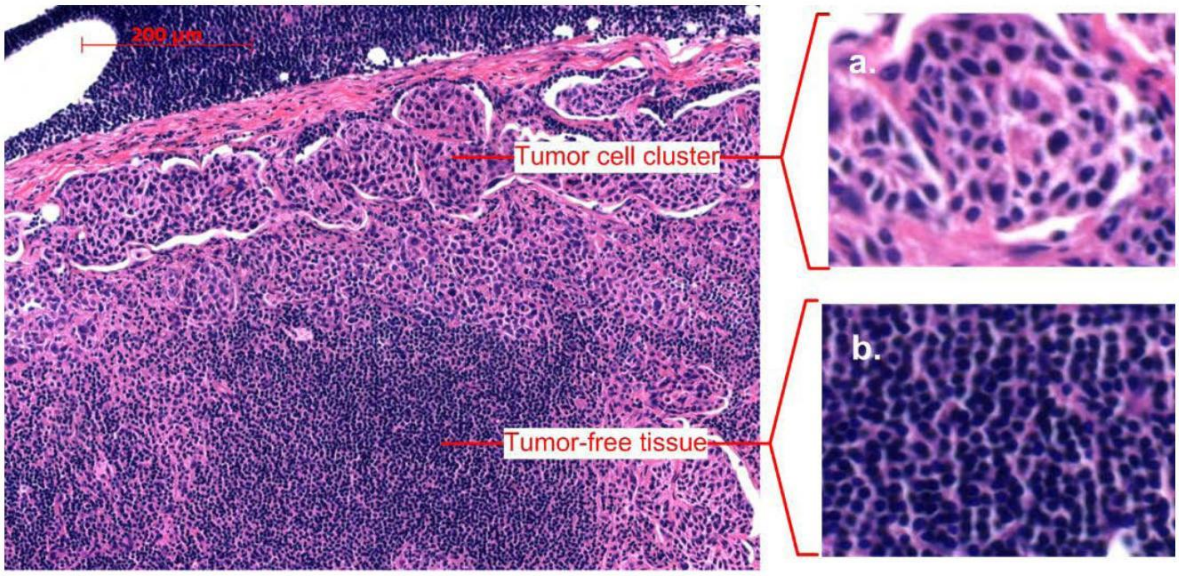



**Figure 16: IHC mechanism for histopathological identification of micrometastases**

Immunohistochemistry is recognized to provide evidence on differentiation. By identifying tumor products (such as enzymes and hormones) it provides information about the functional activity of neoplasms. A range of antibodies has been developed to demonstrate steroid hormone receptors, growth factors, oncogene products and may be useful for managing and assessing the prognosis of patients with gynecological cancer (Benjamin 1995). With the help of various biomarkers, immunohistochemical staining is widely used as a diagnostic method. Specific molecular markers are indicated for particular types of cancer. IHC is also widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue.

Immunohistochemical staining with hematoxylin-eosine (H-E) (Figure 17) is presently used in the routine to diagnose primary or secondary tumors. H-E staining consists of staining with both hematoxylin and eosin. Hematoxylin is a salt that dissociates in water into positive and negative ions. Its positive ion (that is basic, alkaline) readily combines with negatively charged regions of cellular macromolecules, especially phosphate groups of nuclei acids, coloring them in deep blue. Cell nuclei are usually deeply blue or basophilic preferentially staining with hematoxylin because of their high nucleic acid (DNA and RNA) cells. Eosin is also a salt that disassociates in water into ions. Its negative ion, which is an acid in nature, readily combines with positively charged regions of cellular macromolecules, especially the positively charged regions of cytoplasmic proteins coloring them in a variety of colors, ranging from pink to red. Cell cytoplasm is therefore usually pink or eosinophilic (Figure 17). In general, squamous cells such as skin-cells are usually pink or eosinophilic preferentially staining with eosin; however, most cells have a reproducible staining pattern, regardless of the tissue.




Legend:

(a) Tumor cells: cell nuclei: deep blue (basophilic), cell cytoplasm: pink (eosinophilic)

(b) Lymphocytes

**Figure 17: Lymph node metastasis stained for H-E (Enlargement: $20^x$)**

Immunohistochemistry is a multi step procedure that requires selection of appropriate reagents, tissue assortment, good fixation and tissue processing, preparation of slides, usage of appropriate antibodies and good interpretation of staining results. Correct tissue staining depends on the handling and processing of the tissue prior to staining. Inadequate or prolonged fixation of tissue may cause loss of antigenicity. Contamination with other tissues or fluids can cause artifacts, antibody trapping or false-negative results. Moreover, false positive results may be seen due to non-IHC binding of proteins or substrate reaction products. They can also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C) (Benjamin 1995, Lerwill 2004, Miller 2002).

#### 2.3.1.1 Preparation of paraffin-embedded sections

The 120 LN taken from the 48 women are preserved in neutral buffered formalin for routine processing and paraffin embedding. Tissue representative biopsies of a thickness 3-4 mm are fixed for 18-24 hours in neutral buffered formalin. The

tissues are then dehydrated in a series of alcohol and xylene, followed by an infiltration through melted paraffin held at no more than 60°C.

Of each embedded LN tissue of 4-5 µm are prepared and further processed with immunohistochemical staining methods: $p16^{INK4a}$, CK19, AE1/3 and control serum. Counterstaining with Hematoxylin (H-E) enables a better differentiation between the signal molecule and the major structural components of the cell (Campbell 2007); therefore staining with H-E is used to confirm the diagnosis.

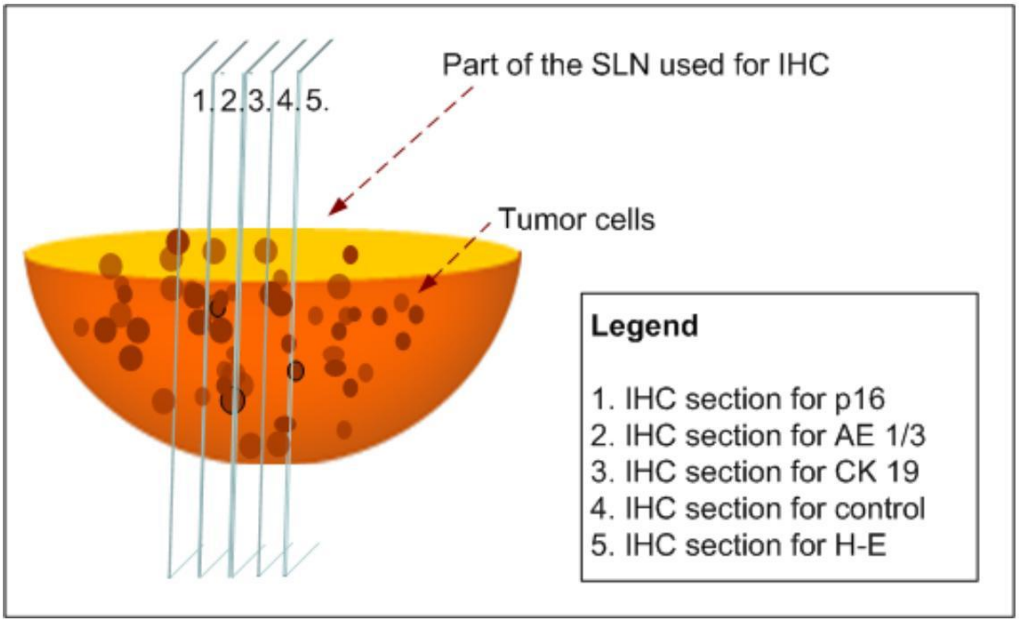



**Figure 18: Serial sectioning for the IHC staining**

#### 2.3.1.2 The procedure for Immunohistochemistry

IHC should be performed by appropriately following the outlined protocol. Deviations from the described protocol might provide false negative results. A specific heat-induced Epitope Retrieval method is recommended before staining the LN tissue. The Epitope Retrieval Solution (ERS) is used to have an optimal assay performance. The Epitope Retrieval should be heated in a water bath. Other methods of heating have been tested but they do not provide satisfying results. This method involves heating of tissue sections mounted on slides that are immersed in the ERS in a calibrated water bath capable of maintaining the ERS at the required temperature 95-99°C. Microwave irradiation is a method employed for the antigen retrieval process. Previous studies report that this

technique is successful in enhancing the range of antibodies that can be used to study formalin-fixed paraffin embedded tissues. The incubation at 95-99°C is done for 40 (±1) minutes, and then the entire jar with slides from the water bath was removed and cooled in the ERS for 20 (±1) minutes at room temperature. For optimal performance, sections were bathed in Wash Buffer for 5 minutes after Epitope Retrieval and prior to staining. Prior to staining, tissues were first deparaffinized in xylol to remove embedding medium and then rehydrated. Deparaffinization and rehydration is done at room temperature (20-25°C). Incomplete removal of paraffin should be avoided as the residual embedding medium results in non-specific staining. During the staining procedure tissues should not dry. Dried tissue sections may present increased non-specific staining. In prolonged incubations, tissues should be placed in humid environment. Because of being fixed before storage, these sections needed no further fixation and were ready for blocking and staining (Campbell 2007); therefore immediately after the Epitope Retrieval performed in a water bath, the staining procedure was commenced (Figure 19). During staining, remaining liquids around the specimen were wiped out by using reagents in a gauze pad.

To avoid staining mistakes, IHC procedure requires well-trained staff, appropriate selection of reagents, LN tissue and tissue fixation and processing, correct preparation of the immunohistochemistry slide, and accurate interpretation of the results.

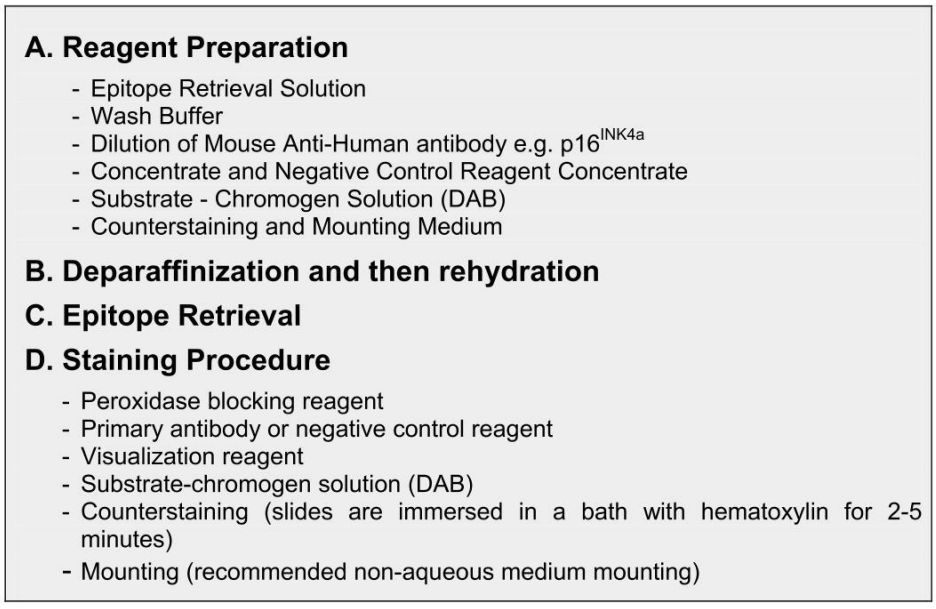



**Figure 19: Specimen preparation by immunohistochemistry**

The information about the antibodies that we used for the IHC technique is summarized in Table 4. The CINtec® Histology Kit of the manufacturer DakoCytomation is an immunohistochemistry assay for the qualitative detection of the $p16^{INK4a}$ antigen on formalin-fixed, paraffin-embedded tissue (DakoCytomation 2010). FLEX Monoclonal Mouse Anti-Human Cytokeratin, Clone AE1/AE3, Ready-to-Use, (Link) and FLEX Monoclonal Mouse Anti-Human Cytokeratin 19, Clone RCK108, Ready-to-Use, (Link) of the manufacturer DAKO (Dako 2010a, Dako 2010b) are intended for use in immunohistochemistry together with Autostainer Link instruments. These two cytokeratin antibodies are used for labeling epithelial cells expressing CK19 or AE1/3 on formalin-fixed, paraffin-embedded tissue sections.

**Table 4: Histology Kits used for the IHC procedure (data taken from (Dako 2010a, Dako 2010b, DakoCytomation 2010))**

| | **AE1/3** | **CK19** | **$p16^{INK4a}$** |
|---|---|---|---|
| **Supplier** | DAKO | DAKO | DakoCytomation |
| **Kit Code No.** | IR053<br>Ready-to-Use; FLEX, for Autostainer Link Instruments | IR615<br>Ready-to-Use; FLEX, for Autostainer Link Instruments | K5334<br>CINTec® Histology Kit $p16^{INK4a}$ |
| **Antibodies used** | Anti-Cytokeratin | Anti-Cytokeratin 19 | Anti-Cyclin-dependent kinase inhibitor $p16^{INK4a}$ |
| **Clone** | AE1/AE3<br>Isotype: IgG1, kappa | RCK108<br>Isotype: IgG1, kappa | E6H4™ |
| **Reagent provided** | Ready-to-use monoclonal mouse Anti-Human antibody provided in liquid form in a buffer containing stabilizing protein and 0.015 mol/L NaN3. | Ready-to-use monoclonal mouse Anti-Human antibody provided in liquid form in a buffer containing stabilizing protein and 0.015 mol/L sodium azide. | Mouse Anti-Human $p16^{INK4a}$ Concentrate antibody, 0.5 mL. Supplied in 0.05 mol/L Tris/HCl, 0.1 mol/L NaCl, 15 mmol/L $NaN_3$, pH 7.2, containing stabilizing protein. |
| **Dilution used** | Diluted EnVisionTM FLEX Wash Buffer (10x), (Link) in room temperature. | Diluted EnVisionTM FLEX Wash Buffer (10x), (Link) in room temperature. | 23 mL, ready to use Tris/HCl buffer containing stabilizing protein and 15 mmol/L $NaN_3$. |
| **Epitope Retrieval** | Necessary | Necessary | Necessary |
| **Storage** | 2-8 °C | | |

### 2.3.1.3 Advantages and disadvantages of immunohistochemistry

IHC is a detection technique that has the advantage of being able to identify where a given protein is expressed within an examined tissue. IHC is a reliable method to examine tissues. However, to avoid false results, a careful application

of IHC protocol is required. In this section we will list the advantages and disadvantages of IHC.

*Advantages of IHC*

- Paraffin wax-embedded fixed tissues are suitable for demonstration of many antigens and so give the greatest information and permit the analysis of archival material (Benjamin 1995, Campbell 2007).
- IHC gives the opportunity to microscopically visualize the architecture and morphology of the tissue
- IHC gives results within a short time (two days)
- There is a broad spectrum of markers that can be used by IHC. These markers are safe and have no side effects such as radioactivity

*Disadvantages of IHC*

- Primary antibodies are not available for all proteins of interest; therefore it can be hard, expensive and time consuming to find a good antibody
- IHC is labor intensive
- IHC procedure involves heating up to 60°C, which could break the antigen bonds formed during fixation, resulting in destroying the antigen, thus, increasing the number of false positive cases. Therefore, in order to prevent damage and drying it is important to correctly monitor the sections during the microwaving process.
- Tissue sample is important. Appropriate fixation and processing of the tissue, proper use of reagents, adequate epitope retrieval, complete removal of paraffin, thorough cleaning of the suitable slides and appropriate reagent incubation time are required in order to avoid false positive and negative results. Therefore, specificity for identifying isolated tumor cells at a single level of LN tissue is questionable (Benjamin 1995, Campbell 2007).

### 2.3.2 PCR technology

Polymerase chain reaction (PCR) has revolutionized the methodology of RNA and DNA detection in pathogenic organisms. It is an in vitro technique that is used to amplify specific regions of DNA strand; these can be a single gene, a part of the gene or a non-coding sequence. The invention of polymerase chain reaction in 1983 is credited to Kary Mullis who, in 1993, was awarded the Nobel Price in Chemistry for his invention (Mullis 1998). Nowadays, the technique is being used to identify DNA sequences, to diagnose genetic diseases, to identify genetic fingerprints, to diagnose infectious diseases and to detect viruses or bacteria and also to study human evolution (Bloom 2009, Max et al. 2001, Max et al. 2002, Molden et al. 2007, Rollins et al. 2000).

In our study, we used the real-time reverse transcription PCR technology to quantify gene expression on histologically positive and negative sentinel lymph nodes. A tissue section of 1-2 mm thickness was taken from each SLN and immediately snap-frozen in liquid nitrogen and stored at -80°C until the HPV RNA extraction (as explained in (Häfner et al. 2007)). RT-PCR is used to amplify, isolate or identify a known sequence from cell culture or tissue derived RNA. The RNA is first reverse transcribed to cDNA which is then amplified by PCR. RT-PCR is widely used in expression profiling to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, if the genomic DNA sequence is known, to map the location of exons and introns in the gene. The 5´ end of a gene (corresponding to the transcription start site) is typically identified by the RT-PCR technique named RACE-PCR (Rapid Amplification of cDNA Ends) (Bloom 2009, Max et al. 2001, Max et al. 2002, Rollins et al. 2000).

#### 2.3.2.1 RNA extraction, reverse transcription and real-time PCR

The RNA extraction, reverse transcription and real-time PCR are done as described by Häfner et al (Häfner et al. 2007). Total RNA is extracted from 30 mg homogenized tissue. The RNA Blood Mini Kit (Qiagen, Hilden, Germany) is used following the manufacturer's instructions. RNA concentration and quality were determined by spectrophotometry and gel electrophoresis. One µg total RNA was reverse transcribed in 20µl using oligo-dT (500 nM) or random primers (200 ng),

dNTP (500 nM each), DTT (10 mM), first strand buffer (Invitrogen, Karlsruhe, Germany), RNaseOUT (20 units) and SuperScriptII reverse transcriptase (200 units) (Invitrogen, Karlsruhe, Germany). All real-time PCR experiments were run on a RotorGene2000 (Corbett Research, Wasserburg, Germany) or on an ABI 7300 SDS (Applied Biosystems, Darmstadt, Germany). Reactions were performed in 25µl volume containing: dNTP (240 lM each), forward and reverse primer (500 nM each), DMSO (5%), MgCl2 (1.75 mM), Tris-HCl pH8.3 (10 mM), KCl (50 mM), gelatine (0.001%) and AmpliTaqGold (1.25 U) (Applied Biosystems, Darmstadt, Germany). Depending on the assay used either SybrGreen (0.25-fold final concentration, Molecular Probes Invitrogen, Karlsruhe, Germany) or a TaqMan probe (200 nM final concentration) was added. For the SybrGreen assays (HPV16 E6, GAPDH, and HPRT) the PCR steps were as follows: Initial denaturation and hot start activation at 95°C for 10 minutes followed by 40 cycles of denaturation phase at 95°C for 15 seconds, primer specific annealing for 20 seconds at different temperatures and elongation at 72°C for 40 seconds. Subsequently, the melting temperature of the PCR product was determined to ensure specificity. All real-time PCR results were quantified using the relative standard curve approach (Giulietti et al. 2001). Serial dilutions from 10 to 106 copies of plasmid cloned target sequences (pCRII-TOPO, Invitrogen, Karlsruhe, Germany) were used as standard curves. Of each cDNA housekeeping gene and target gene expression was determined. According to Vandesompele and colleagues (Vandesompele et al. 2002), a set of 8 genes tested in 20 SLN samples were used to evaluate the gene expression stability of housekeeping genes in lymph nodes (Häfner et al. 2004). Target gene expression measured in duplicate was normalized to the geometric mean of the expression of the two most stable housekeeping genes in lymph nodes (HPRT, GAPDH).

#### 2.3.2.2 Performance of qRT-PCR assays

Quantitative PCR is an established and reliable method permitting the sensitive and specific detection of DNA or RNA species. We have established a SybrGreen assay for HPV16 oncogenes, termed HPV16 E6, which detects all transcript species encoding the oncogenes E6 and E7 or E7 only. The amplicon is located at the 5´ end of the viral transcript. Because cDNA synthesis is done with oligo-

dT, our approach allows the detection of mRNA only, which reflects the presence of intact occult tumor cells (Häfner et al. 2007). Single tumor-cells can be detected by use of RT-PCR; furthermore one tumor cell can be detected in a background of $10^{6}$-$10^{7}$ normal cells.

#### 2.3.2.3 Advantages and disadvantages of PCR technique

PCR technology is possible to take a sample of genetic material even from one single cell, copy its genetic sequence repeatedly, and produce a test sample that is sufficient to discover the presence or absence of a specific virus, bacteria or every specific sequence of genetic material. Although time and labor consuming, PCR is a reliable method that has a high sensitivity, accuracy and precision. The advantages and disadvantages of the technique are listed as follows:

*Advantages of PCR techniques*

- PCR is a reliable test that makes possible to amplify any small segment of DNA (e.g. smaller than 5kb). PCR methods are very sensitive and able to detect a wide range of HPV types and its DNA at very low or a single copy level and even at an overwhelming background of human DNA. A tumor cell can be evidenced in a background on $10^{6}$ – $10^{7}$ HPV negative cells.
- Because of the high sensitivity of PCR methods, detecting the DNA of pathogenic agents (such as viral pathogens) may be possible soon after infection starts and before the clinical symptoms appear. PCR tests detect the pathogenic agents earlier than serologically-based methods, while patients can take weeks to develop antibodies against an infectious agent. The amount of virus in the sample can be quantified by quantitative RT-PCR techniques (see next advantage of PCR). Earlier detection of infection can mean earlier treatment and an earlier return to good health (Max et al. 2002).
- The test offers a high level of accuracy and precision. Quantitative RT-PCR allows not only the identification of rare transcripts but also quantification of amounts of DNA, cDNA or RNA. Additionally to determining whether DNA sequence is present in the sample tissue, it

gives information about the number of its copies in the sample (Max et al. 2001, Max et al. 2002, Rollins et al. 2000).

*Disadvantages of PCR techniques*

- No morphological correlation is possible
- The procedure is time consuming and labor intensive. Real-time PCR requires an instrumentation platform that mainly consists of a thermal cycler where the process takes place, an instrument that automatically controls and alternates the temperatures for programmed periods of time for the appropriate number of PCR cycles (usually between 30 and 40 cycles), computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software
- It is important that RNA samples are free of DNA contamination; otherwise PCR might fail causing amplification of false DNA products.

### 2.3.3 Microscopic evaluation

The evaluation of the micrometastases stained by the IHC markers $p16^{INK4a}$, CK19 and AE1/3 is done by using a computer-assisted light microscope. One or two serial sections of the formalin fixed paraffin embedded SLN tissue were stained for each marker: $p16^{INK4a}$, CK19 and AE1/3 (Figure 20). Different microscopic enlargements ($5^x$, $10^x$, $20^x$ and $40^x$) were used to detect the tumor cells or clusters, to measure their dimensions and to note the parameters of their localization in the SLN.

The microscope has an installed camera that allows taking pictures of the tissue at different enlargements. A computer that is connected to the microscope allows tissue images to be shown on the screen and pictures taken during microscopy can be saved for later usage.

**Figure 20: Two serial sections of the SLN stained by IHC**

IHC allows indicating the geographical location of positive cells or groups of cells (cell clusters) along with other cells of the same and of different phenotype within the framework of the overall architecture of the tissue (Campbell 2007). Coordinates of isolated and distributed occult tumor cells classified under Group B, C and D as well as the parameters of micrometastases and metastases classified under Group A are measured and entered into an Access database (Paragraph 2.4). The microscopic results are confirmed by at least an expert pathologist.

#### 2.3.3.1 Advantages and disadvantages of microscopy

Microscopy by using a light microscope uses visible light transmitted through or reflected through a single or multiple lenses to allow a magnified view of the sample tissue. The technique can only image dark or strongly refracting objects. Microscopic evaluation requires skilled experts and appropriate lightening; however, there are no major disadvantages of microscopy.

*Advantages of microscopy:*

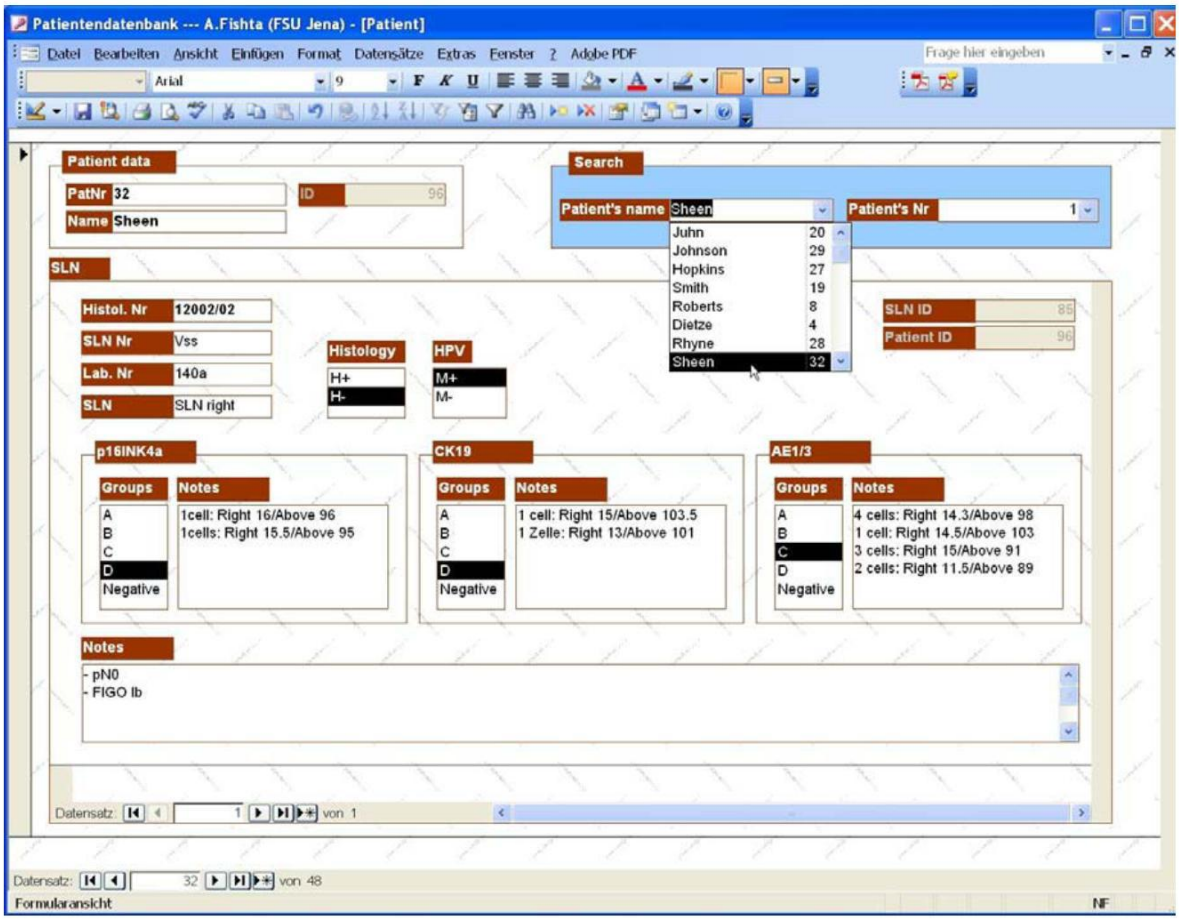
- Detection of tumor clusters or cells is easy when the IHC staining procedures are appropriately performed
- Microscopy is a cheap method which only requires a good light microscope, concentration and evaluation of the whole lymph node tissue for the presence of micrometastases or occult tumor cells

*Disadvantages of microscopy:*

- Possible errors during evaluation
- Labor consuming
- For reliable results, a selection of appropriate markers is necessary
- As it is performed in the routine, one to two sections are not representative of the entire tissue, which may lead to false results of microscopic evaluation.

## 2.4 Data storage and management

Data of each patient and her SLN are stored in a Microsoft Access database (Appendix 2). For each LN, outcomes of microscopic evaluation including parameters of cluster micrometastases as well as coordinates of localization of tumor cells are recorded in the database. The main form used to enter the data is illustrated in Figure 21. The patients´ names shown in this figure are not true, real names are confidential. The histological number (Histo Nr) is unique for each patient and is used as a primary key according to the database concepts. The histological number comprises the personal code of the patient and the year when she underwent surgical treatment for example: “4479/04” means that “4479” is the patient-code and that in year 2004 the patient went under surgery. In the form microscopic results for the three IHC markers: $p16^{INK4a}$, CK19 and AE1/3 are entered as well (Appendix 2). The database also contains the results of: Conventional histology of the SLN, RT-PCR for presence or absence of HPV mRNA, and additional data about the primary tumor (e.g. HPV-type) or the patient.




The main data elements considered in this study are (a) Re-sectioning for H-E staining of SLN: Positive conventional histology is marked “H+” and negative histology is marked “H-”; (b) HPV mRNA: HPV mRNA positive is marked “M+” and HPV mRNA negative is marked “M-”; (c) Parameters and localization of micrometastasis or tumor cells seen in a microscopic view with enlargement $5^x$, $10^x$, $20^x$ and $40^x$ help to easily re-find them microscopically; (d) Notes: This field serves to enter additional data for each specific lymph node. These data might help to interpret better the results or could be needed for later use.

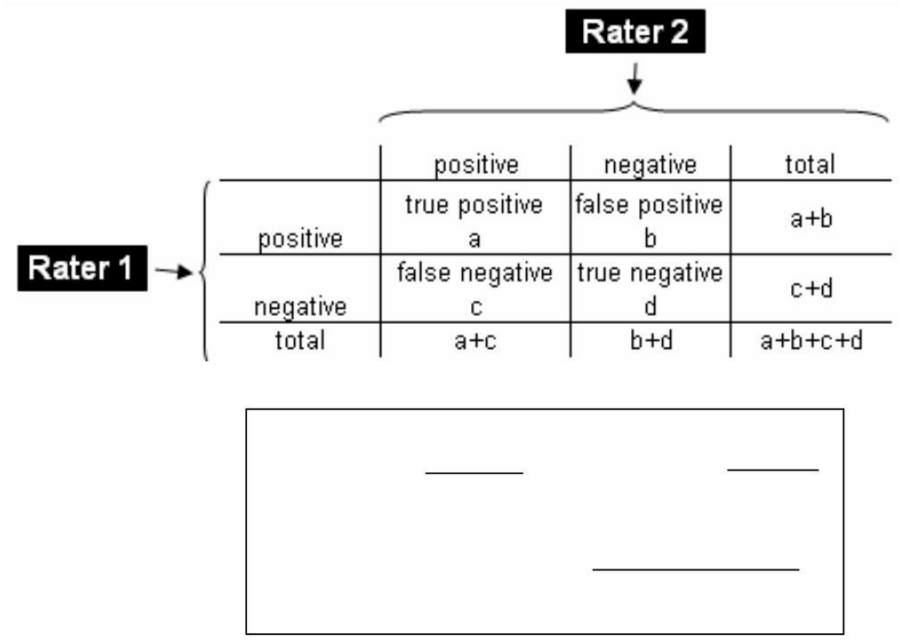
**Figure 21: A MS Access form for acquisition of study-relevant data**

## 2.5 Statistical methods

The validation of the markers can be performed by the use of descriptive statistics. The “two by two” Table of Agreement can be used to measure the agreement between the markers and to calculate the sensitivity, specificity and overall agreement (Paragraph 2.5.1). The Cohen’s Kappa Coefficient of Agreement can be used to re-confirm the agreement of markers (Paragraph 2.5.2).

### 2.5.1 "Two by two" Table of Agreement

The "two by two" Table of Agreement can be used to determine the sensitivity, specificity and the extent of the agreement between two raters. Sensitivity and specificity are constantly used in validity tests. Sensitivity is the probability that the true positive cases are detected; specificity is the probability that the true negative cases are detected and the overall agreement expresses the percentage of present agreement between the two raters (Rater1 and Rater2).




"a" and "d" represent the number of times the two raters agree; "b" and "c" represent the number of times the two raters disagree. If there is no disagreement between rater 1 and 2, which means that "b" and "c" are zero; then the sensitivity and specificity are 100%, consequently, the overall agreement is also 100%. In this case there is a perfect 100% agreement between the two raters indicating that the test is excellent. If "a" and "d" are zero, between the two raters there is no agreement at all. Consequently, the sensitivity, specificity and the overall agreement are also zero.

**Figure 22: "Two by two" Table of Agreement, sensitivity, specificity and overall agreement**

Figure 22 illustrates the way the "two by two" Table of Agreement is presented and how the sensitivity, specificity and overall agreement are calculated. In our study, the "two by two" Table of Agreement was used to measure the extent to which the IHC markers agree between each other (Paragraph 3.3.1) as well as how much each IHC marker agrees with the molecular marker HPV mRNA (Paragraph 3.3.2).

### 2.5.2 Cohen's Kappa Coefficient of Agreement

"Cohen's Kappa Coefficient" (Cohen 1960) statistically measures the agreement between the two raters when both are rating the same object. Cohen's Kappa can be affected by prevalence; therefore, it might not be appropriate to compare Kappa results between studies or populations. Nonetheless, it can provide more information than a simple calculation of the row proportion of agreement (Bortz and Lienert 2003, Hsu and Field 2003, Viera and Garrett 2005). Cohen's Kappa Test is only available for tables that have the same categories in the columns and rows. Therefore, it is applicable for the "two by two" Tables (Cicchetti and Feinstein 1990). We used it to re-confirm the agreement between the four target tumor markers (Paragraph 3.3.3). As a statistic test, Kappa could determine whether agreement exceeds chance levels.

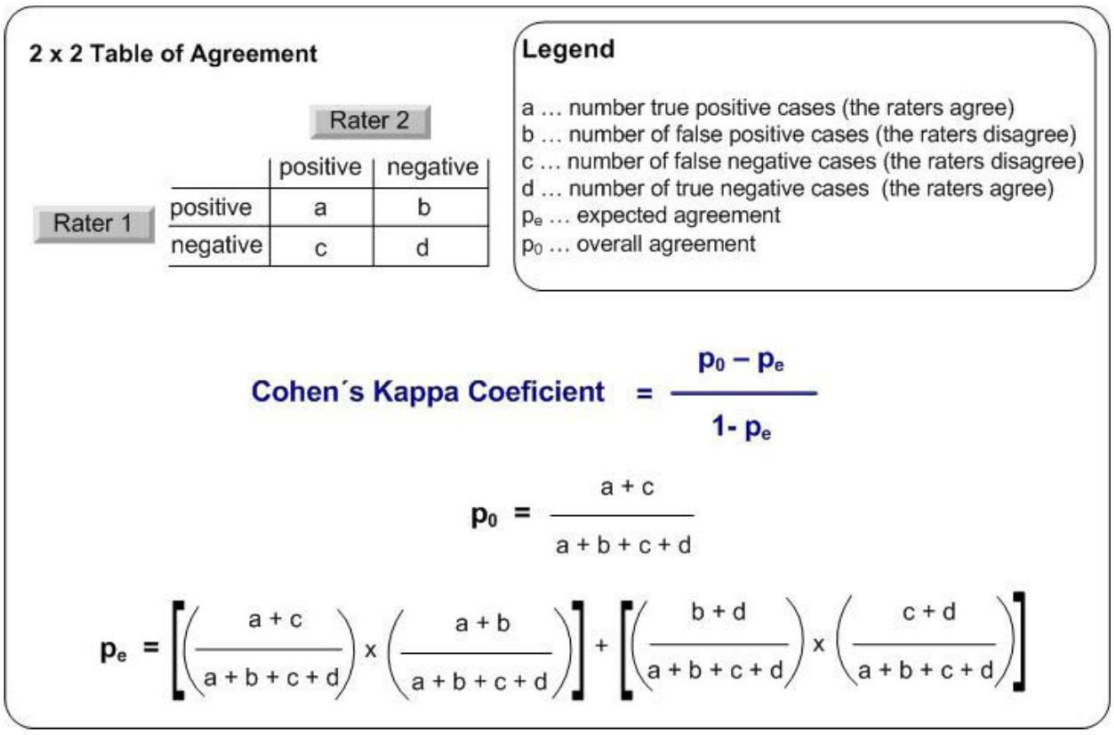



**Figure 23: Computation of the Cohen's Kappa based on the "two by two" Table of Agreement**

Cohen's Kappa statistic can be used as:

- A statistics test to analyze the rater independence. It involves testing the null hypothesis; that there is no more agreement that might occur by chance given random guessing. This is a qualitative use of Kappa with

"yes" and "no" decision about whether raters are independent or not, which is actually not very informative.

- A way to quantify the level of agreement (as illustrated in Figure 24).

Kappa value, which is based on the "two by two" Table of Agreement, is calculated as shown in Figure 23. Computation of the Kappa agreement depends on the values of the overall agreement ($p_0$) and the expected agreement or the proportion of chance ($p_e$). $p_e$ is the proportion of times raters would agree by chance. However, this term is relevant only when the two raters are statistically independent from each other. As raters are obviously independent, the relevance and appropriateness of this term as a correction to actual levels of agreement is quite questionable.

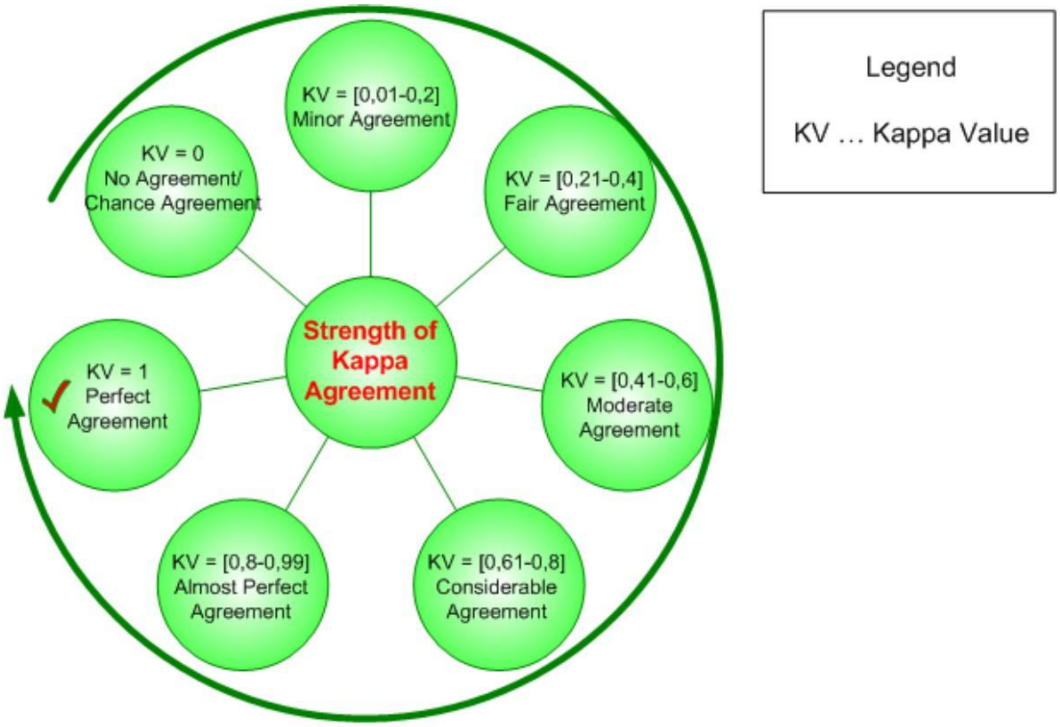



**Figure 24: Cohen's Kappa Agreement**

As illustrated in Figure 24, the Cohen's Kappa agreement is smaller than the "chance" agreement if the value of Kappa results to be smaller than zero (<0). This situation indicates an application problem. When values of Kappa fall between 0.01 – 0.20 it means that there is a "minor agreement" between the two raters. Furthermore, the agreement is "fair" when Kappa results between 0.21 and 0.40; it is "moderate" if Kappa values fall between 0.41 and 0.60. For Kappa values between 0.61 and 0.80 the agreement is "considerable" and if Kappa

results between 0.81 and 0.99 the agreement is “almost perfect”. This indicates that the “perfect” agreement equates to Kappa equal to 1 and the “chance” agreement equates to Kappa equal to 0 (zero). Consequently, the value “0” of Kappa indicates that agreement is not better than chance (Landis and Koch 1977, Viera and Garrett 2005).

# Chapter 3: Results

As described in the previous chapters, lymph nodes are filters along the lymphatic system. They filter out and trap bacteria, viruses, or cancer cells that travel through the body via the lymph fluid. Particularly SLN play an important role in the prognosis of cancer because they are the hypothetical first lymph nodes reached by tumor cells that migrate from the primary tumor via the lymphatic system. In this study, 120 SLN (85 pN0, 35 pN1) from women with primary CCa were analyzed for metastases, micrometastases and occult tumor cells by using three IHC markers ($p16^{INK4a}$, CK19 and AE1/3) and a molecular marker (HPV mRNA). These markers are used in research studies to diagnose a number of cancer entities, but their reliability for detecting metastases, micrometastases, tumor cell clusters or single tumor cells in lymph nodes of patients with CCa has not yet been analyzed in a comparative study.

To test the staining quality of the selected IHC markers, 35 histologically positive SLN (pN1) were re-sectioned and stained for the three markers: $p16^{INK4a}$, AE1/3 and CK19. Subsequently, their staining patterns in micrometastases and metastases were evaluated (Paragraph 3.1).

The 85 SLN that were originally negative by histopathology (pN0) were re-evaluated for the tumor presence by the help of three IHC and one molecular marker. The agreement between the IHC markers was measured (Paragraph 3.3). AE1/3 provided the best staining quality; therefore, it is chosen as our “gold standard”. Each IHC marker was then compared to the molecular marker HPV mRNA (Paragraph 3.3.2), which is also a potential marker for detecting LN tumor presence in patients with primary CCa. The statistical evaluation is done with the help of the “two by two” Table of Agreement and the Cohen’s Kappa statistics.

Sensitivity, specificity and overall agreement between the selected markers are used to measure the validity of the selected markers. The calculations were done by the use of the statistical software SPSS 12.

The existing TNM classification was extended in order to be able to consider the lymph node occult tumor cells and tumor cell clusters that are smaller than 0.2mm. The new classification contains four positive groups (A, B, C and D) and a negative group (Paragraph 3.2). Using this classification we were able to differentiate the tumor positive LN with regard to the tumor size or the number of detected tumor cells. Based on statistical outcomes, we concluded about the reliability on the four proposed markers for detecting tumor clusters and occult tumor cells in lymph nodes.

## 3.1 Marker gene expression in pN1 lymph nodes

Among the 120 evaluated SLN, 85 were negative (pN0) and 35 were positive (pN1) by the conventional histopathological examination. After a renewed sectioning, metastases or micrometastases were found in 31 of histologically positive SLN (88.6%). The re-sectioning of the histologically positive SLN was done for the following reasons:

- to validate the staining protocol of the three IHC markers (AE 1/3, $p16^{INK4a}$ and CK19)
- to evaluate the heterogeneity of the staining pattern within the tumor clusters (metastases or micrometastases)

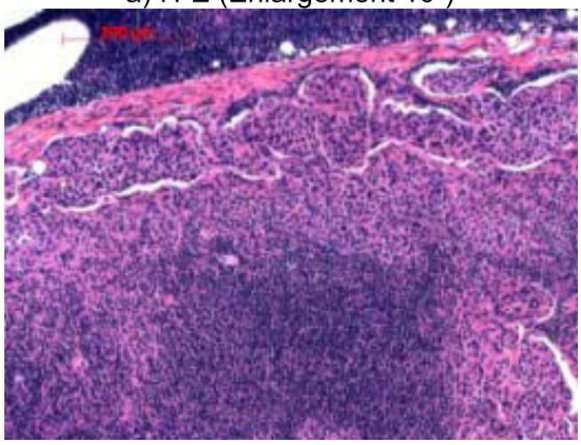
### 3.1.1 Validation of the staining protocol of AE1/3, $p16^{INK4a}$ and CK19

Conventional histopathological examination with hematoxylin-eosin (H-E) is used to diagnose metastases or micrometastases positive tissues. H-E or a tumor specific IHC marker stains tumor cells or clusters and makes them easily distinguishable from the tumor-free tissue. The H-E staining is, nowadays, the most conventional stain for formalin-fixed paraffin sections. Therefore, to validate the staining protocol of our selected IHC markers ($p16^{INK4a}$, CK19 and AE1/3), the serial sectioned SLN tissue was stained with H-E as well. Then the staining

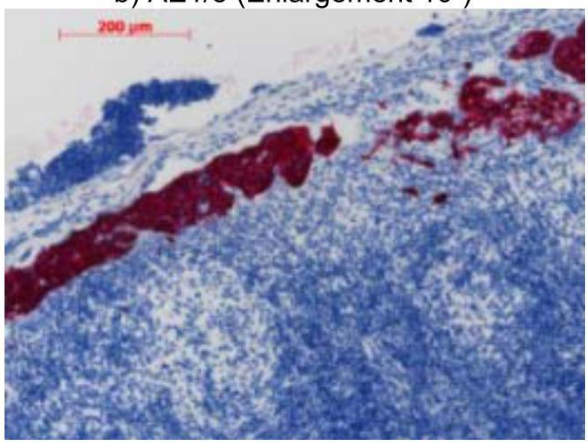
quality of metastases or micrometastases detected by $p16^{INK4a}$, CK19 and AE1/3 was compared to the conventional H-E staining (Figure 25 and Figure 26).

Based on changes in cell morphology, hematoxylin and eosin stained sections are used in the routine to make the histopathological diagnosis. Tumor cells differ from the other cells in size, shape, structure, growth rate and function. Their morphology varies from the parent cells: Parent cells are more or less of the same shape and size and have a small nucleus located in the same place of each cell. On the other side, cancer cells have usually different sizes and shapes and their nuclei are larger than at normal cells and are positioned in different places of each cell. Cancer cells are characterized by an uncontrolled growth and loss of structural cell integrity. They may utilize existing mechanisms that support continuous growth while lacking responses to inhibitory mechanisms that control growth in normal cells (Kumar et al. 1999, Siegfried et al. 2000). Functional changes are reflected on the structural level and, consequently, on the overall organization of the tissue. The cell organelles including the nucleus usually increase their size. Comparing to the other cells of the same tissue, cell transformations include nuclear enlargement with increased density and abnormal consistency. These morphological characteristics make the tumor cells distinguishable by staining with the conventional H-E or other tumor specific markers. By means of histopathology (H-E) they are stained in blue-pink color (Figure 25-a, Figure 26-a) (see also Paragraph 2.3.1). Accordingly, the nuclei and ribosome of the cells are stained bluish and the cytoplasm pink. Additionally, the collagen that is abundant in the matrices of most connective tissues is distinctly eosinophilic or pink.

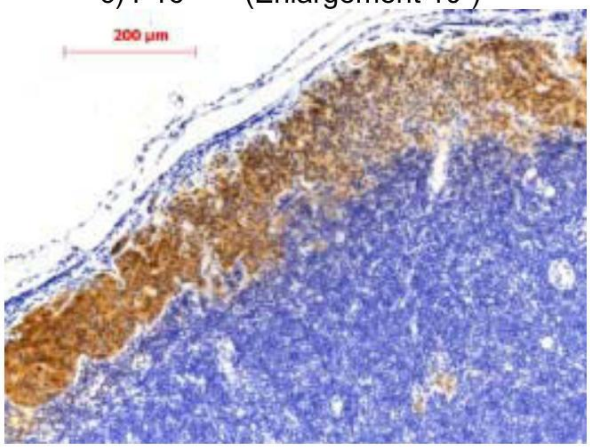
a) H-E (Enlargement $10^x$)



b) AE1/3 (Enlargement $10^x$)




c) $P16^{INK4a}$ (Enlargement $10^x$)




d) CK19 (Enlargement $10^x$)

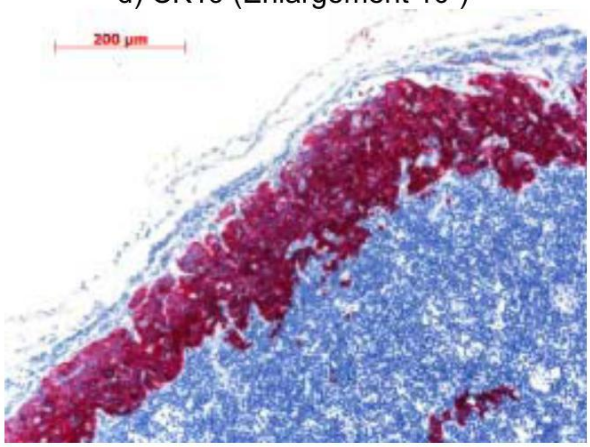
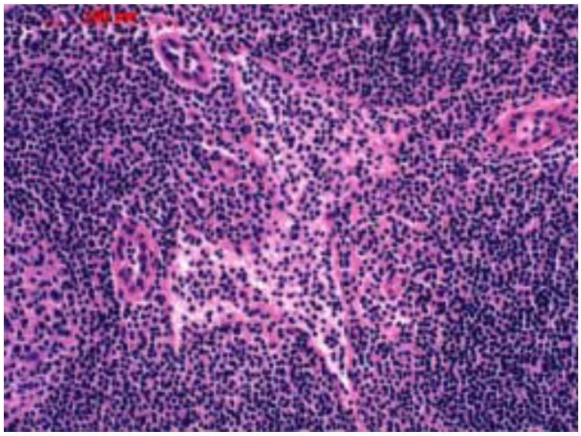



**Figure 25: A SLN metastasis ($10^x$ enlargement)**

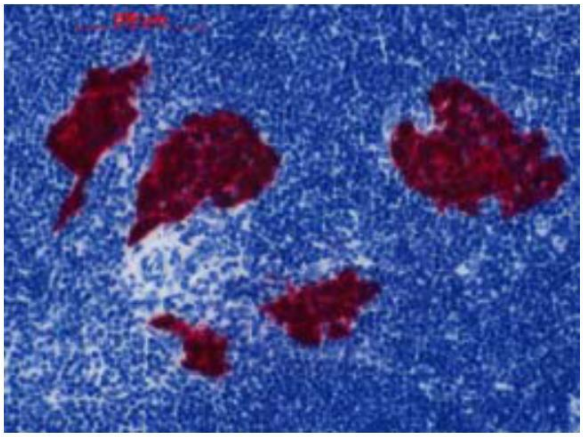
Renewed serial sectioning of the paraphine maintained SLN tissue is performed and stained respectively for H-E, $p16^{INK4a}$, CK19 and AE1/3. After re-sectioning of the 35 originally histologically positive SLN (pN1), the 31 pN1 SLN that resulted micrometastasis or metastasis positive by IHC (H-E, AE1/3, $p16^{INK4a}$ and CK19) were used to validate the staining quality for the three selected IHC markers. These markers stained tumor cells in dark brown ($p16^{INK4a}$) or pink (CK19 and AE1/3). The malignant cells are microscopically counterstained on a white background where the lymphocytes of the non-cancerous SLN tissue stained blue. Figure 25 and Figure 26 illustrate the staining for H-E, AE1/3, $p16^{INK4a}$ and CK19 of two different metastases using two different microscopic enlargements (respectively $10^x$ and $20^x$). Micrometastases shown in these figures are easy identifiable by H-E staining. Moreover, they are also homogeneously stained by

AE1/3, p16$^{INK4a}$ and CK19 and can be well differentiated from the non-tumor tissue (the blue lymphocytes).

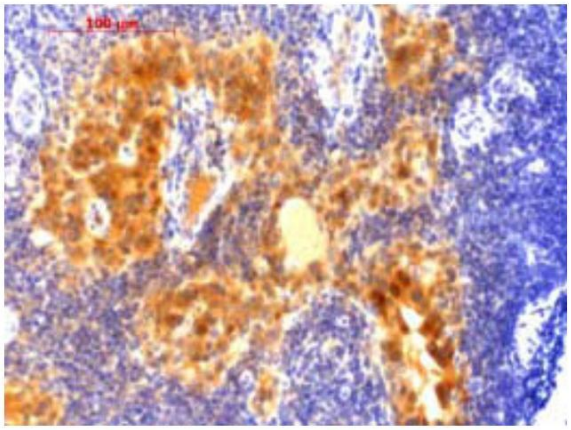
a) H-E (Enlargement 20$^{x}$)



b) AE1/3 (Enlargement 20$^{x}$)



c) P16$^{INK4a}$ (Enlargement 20$^{x}$)



d) CK19 (Enlargement 20$^{x}$)

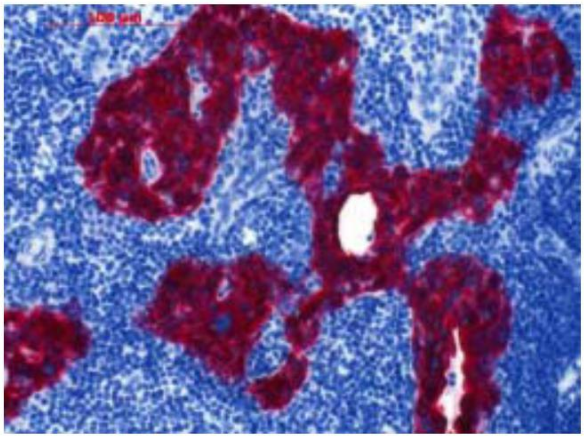


**Figure 26: A SLN metastasis (20$^{x}$ enlargement)**

### 3.1.2 Evaluation of the homogeneity of the staining pattern of the IHC markers in SLN micrometastases and metastases

The staining pattern reflects the intensity and distribution of antigen staining in specimens. Homogeneity of the staining pattern is important for the detection of tumor cells or clusters. Heterogeneous staining might result in interpretation errors and consequently in a number of SLN incorrectly diagnosed as negative. Staining quality of micrometastases or metastases detected by histopathology (H-E staining) and IHC (p16$^{INK4a}$, CK19 and AE1/3) in serial renewed sectioning of SLN were microscopically evaluated. It was noticed that the staining intensity for

AE1/3 and $p16^{INK4a}$ was homogeneous in all micrometastases and metastases, whereas CK19 provided a both heterogeneous and homogeneous staining. In the cases where CK19 stained homogeneously, the cytoplasm of tumor cells was stained in pink, clearly differentiating the tumor cells or clusters from the tumor-free tissue (as illustrated in Figure 25-d and Figure 26-d). However, in several SLN, fractional staining pattern for CK19 was evidenced within the same micrometastasis or metastasis. In addition to that, in some lymph nodes CK19 failed to stain entire micrometastases or metastases that were stained for the other IHC markers and that, due to the morphology of tumor cells, were also evident in the section stained for CK19. Figure 27 and Figure 28 illustrate two examples of heterogeneous staining for CK19 (biopsy A and B) which are compared to the homogeneous staining for H-E, AE1/3 and $p16^{INK4a}$.

**Biopsy A: SLN Nr. 1589, Enlargement $5^x$**

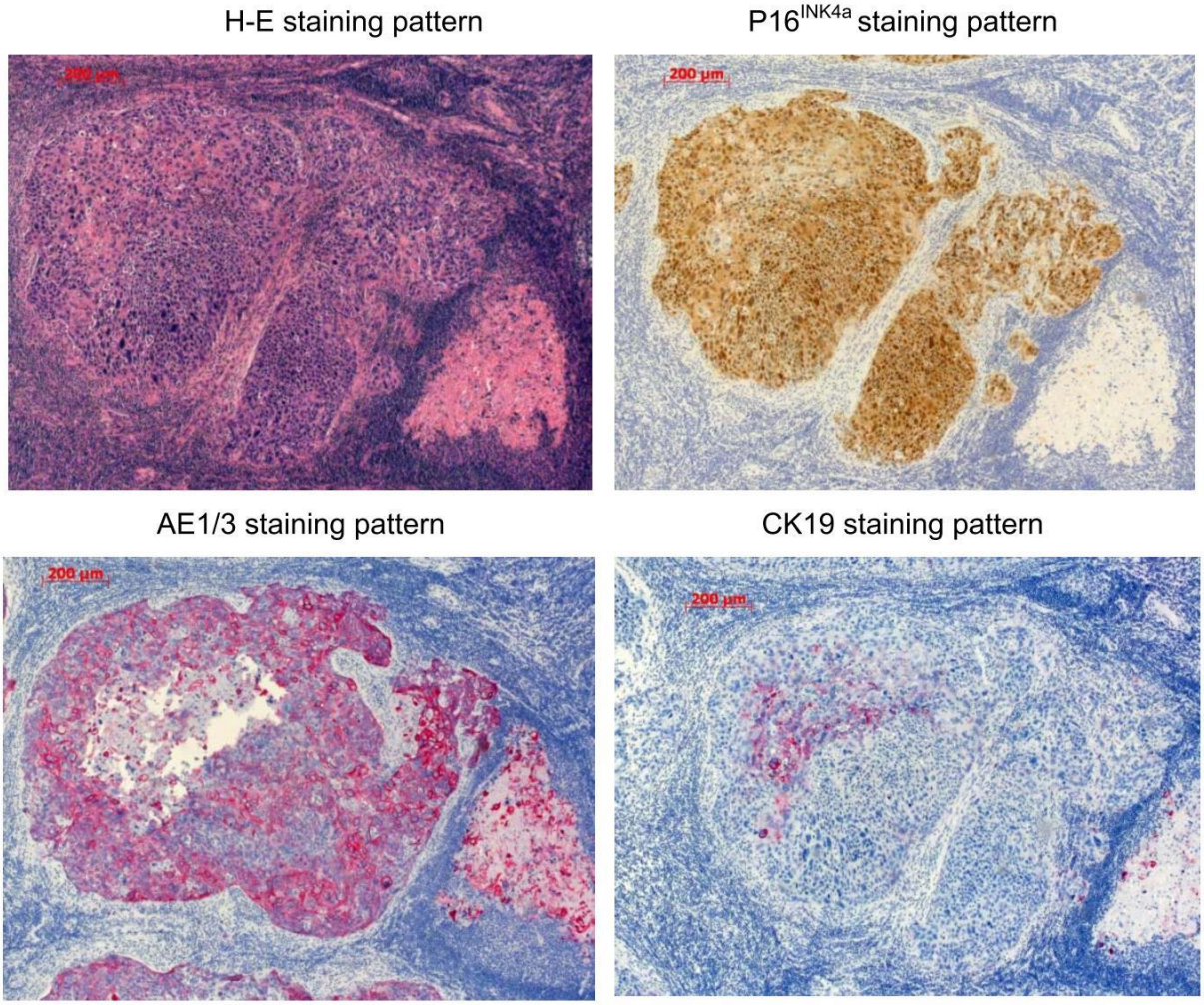



**Figure 27: Heterogeneous staining pattern for CK19 compared to staining for H-E, $p16^{INK4a}$ and AE1/3 (Biopsy A)**

**Biopsy B: SLN Nr. 12985, Enlargement 10$^{x}$**

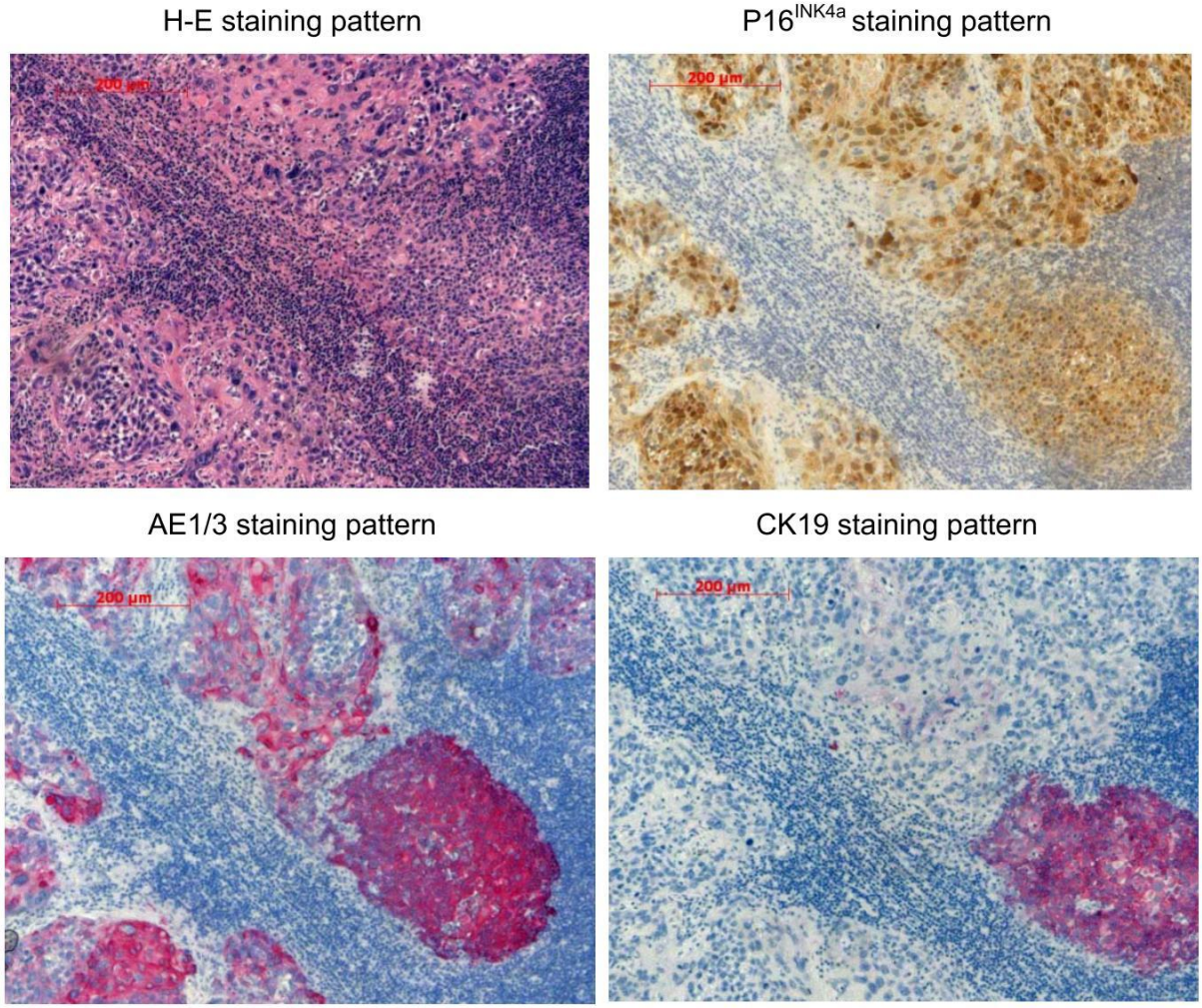


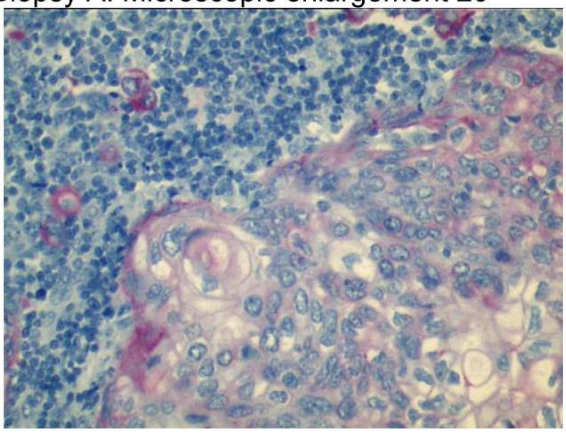

**Figure 28: Heterogeneous staining pattern for CK19 compared to staining for H-E, p16$^{INK4a}$ and AE1/3 (Biopsy B)**

There were also SLN where micrometastases or metastases stained homogeneously for CK19 but in weak color (pink). Figure 29 illustrates several metastases with weak staining intensity by CK19.

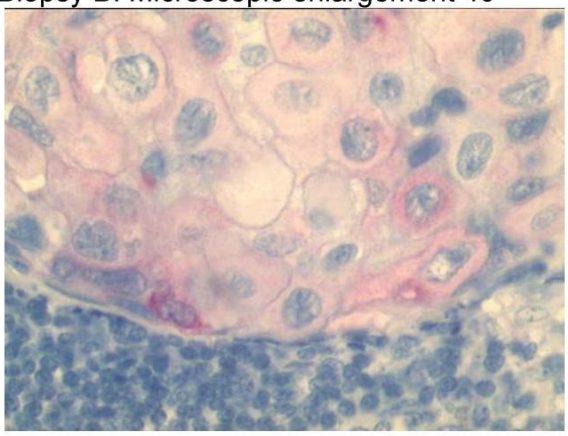
Even though staining of LN metastases and micrometastases for CK19 is often weak and heterogeneous, we expected that this marker would still detect all tumor clusters larger than or equal to 0.2mm (micrometastases and metastases). In fact, among the 31 pathological positive SLN (pN1), all micrometastases and metastases stained for AE1/3 and p16$^{INK4a}$ but they failed to be stained by CK19 in one SLN. Pictures of two different enlargements of this tumor cluster are illustrated in Figure 30. The present micrometastasis is relatively small; it covered about 2% of the SLN tissue, but has, however, a diameter larger than 0.2mm.

This micrometastasis is evidently stained homogeneously for AE1/3 and $p16^{INK4a}$ (Figure 30-c, d) and is present also in the section stained for CK19 (indicated with arrows), except that it was not stained (Figure 30-a, b). Due to the morphology of its tumor cells, the micrometastasis is distinguishable from the rest of non-malignant lymphocytes in the lymph node. Comparing to the lymphocytes, the tumor cells have bigger size and different shapes. Their cytoplasm has abnormally changed its size and their nuclei are located in different positions of the cells.

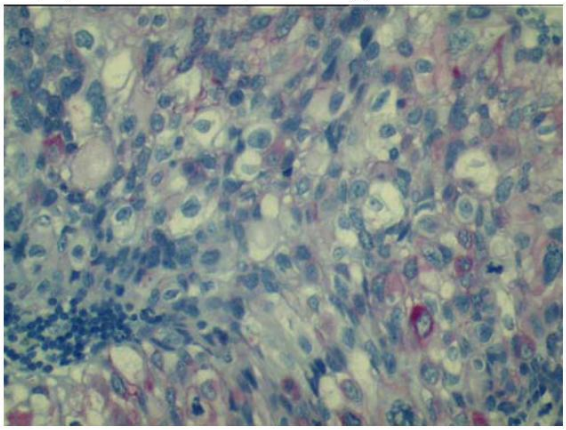
Biopsy A. Microscopic enlargement $20^x$



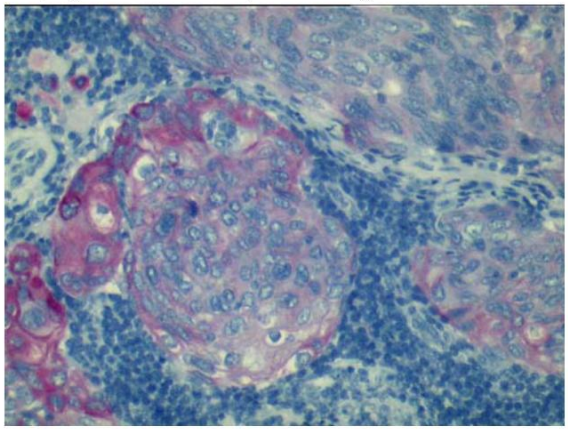
Biopsy B. Microscopic enlargement $40^x$



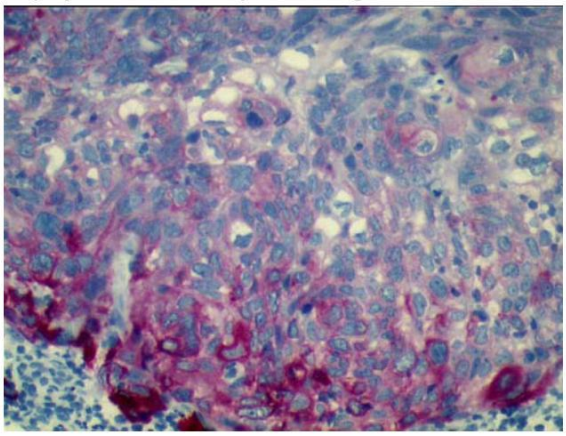
Biopsy C. Microscopic enlargement $20^x$



Biopsy D. Microscopic enlargement $20^x$



Biopsy E. Microscopic enlargement $20^x$



Biopsy F. Microscopic enlargement $10^x$

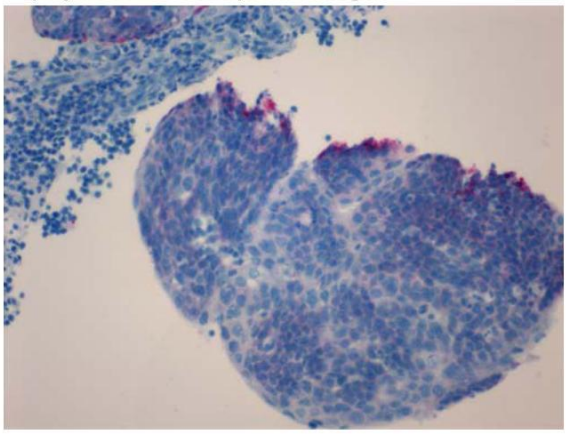
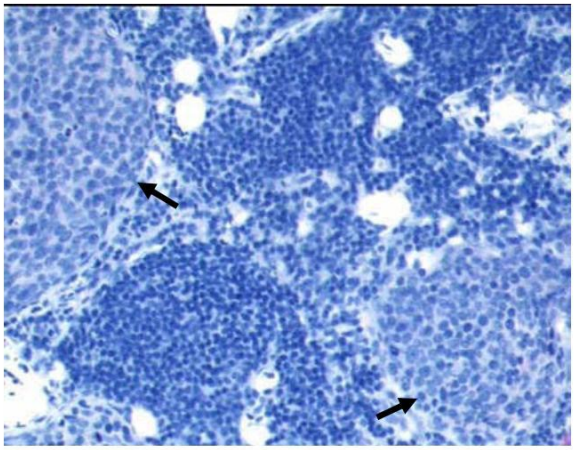
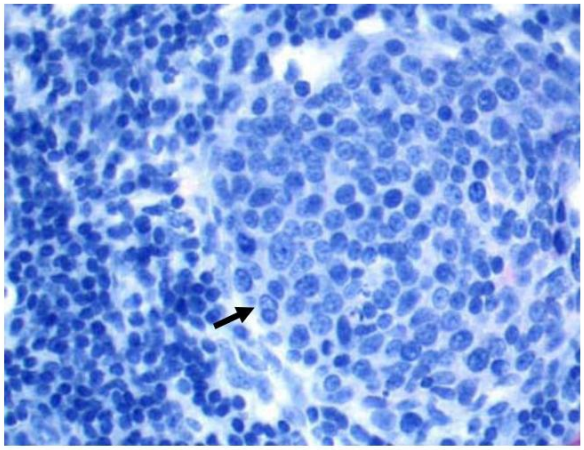


**Figure 29: Weak staining patterns for CK19**

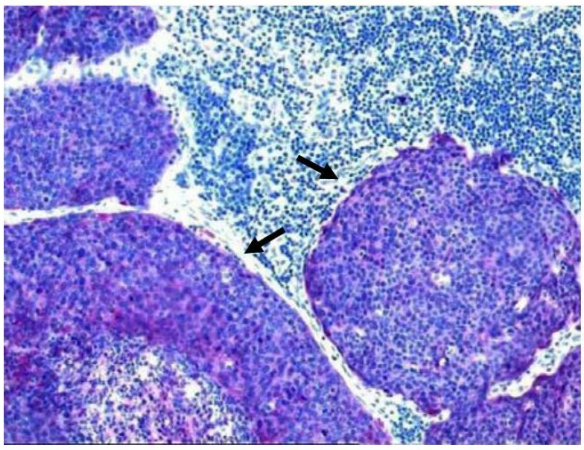
a) Staining for CK19 (Enlargement $20^x$)



b) Staining for CK19 (Enlargement $40^x$)



c) Staining for AE1/3 (Enlargement $10^x$)



d) Staining for $p16^{INK4a}$ (Enlargement $20^x$)

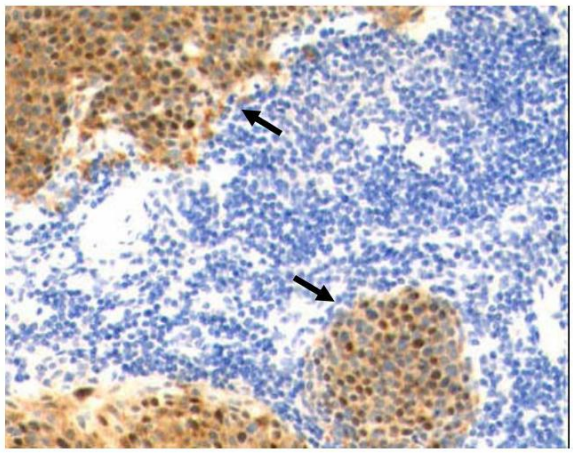


**Figure 30: Microscopic staining of tumor clusters missed by CK19**

Based on these findings, we consider that the reliability on CK19 as a potential marker for lymph nodes is questionable. However, to statistically measure the reliability on CK19, in the following paragraphs of this chapter we will evaluate how much CK19 statistically agrees with AE1/3, $p16^{INK4a}$ and HPV mRNA for the detection of tumor cells and clusters in SLN. Consequently, it will be discussed whether CK19 should be excluded as a potential marker for LN.

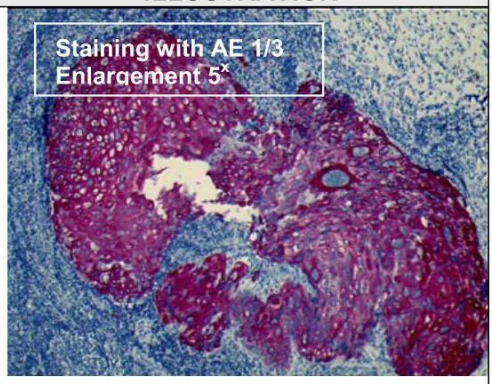
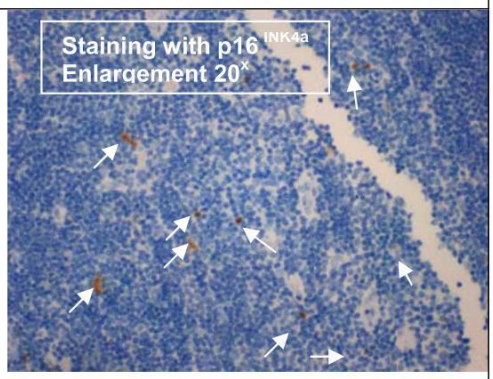
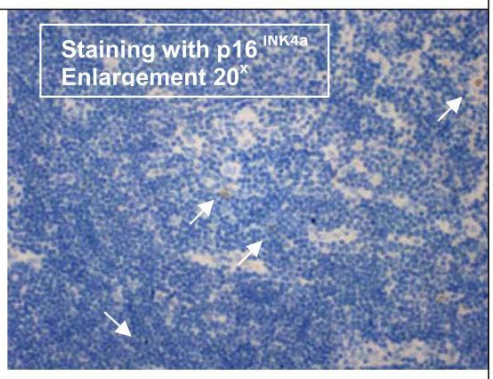
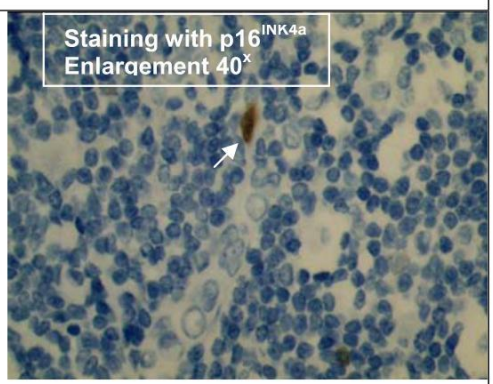
## 3.2 Proposed classification for tumor cells and clusters in LN

The presence of lymph node micrometastases or metastases (pN1 status) correlates significantly with decreased survival and recurrence of patients treated for CCa. According to the American Joint Committee on Cancer Staging for Breast Cancer (AJCC), micrometastases are considered to be structured tumor

clusters with diameter larger than 0.2mm but smaller than 2mm while metastases are tumors larger than 2mm (AJCC 2002b). As explained in the previous chapters, along with other prognostic factors for CCa (tumor grade, lymph-vascular space involvement, surgical margins and distant metastases in other organs), the lymph node status is highlighted as the most important parameter for disease outcome. According to the TNM classification (Paragraph 1.2), patients with primary cervical cancer and pN0 status are supposed to have good prognosis after the primary CCa is treated. However, it is seen that 15% of patients with primary squamous cell carcinoma of the cervix (FIGO stage IB) and tumor-free lymph nodes (pN0) suffer disease recurrence after being treated for the primary tumor (Delgado et al. 1990). Although occult tumor cells might be present in lymph nodes, these patients are still considered to have pN0 status. Distant LN occult tumor cells or tumor cell clusters with dimensions smaller than micrometastases (<0.2mm) could be the reason for poor prognosis of patients with CCa. Several studies are, therefore, questioning the potential prognostic role of occult tumor cells in lymph nodes.

In this study, we expanded the TNM classification into a classification that consists of five categories, four of which (Groups A, B, C and D) include lymph nodes that are positive for metastases, micrometastases, tumor clusters or occult tumor cells and one category (Group Negative) represents lymph nodes with no tumor evidence. In Group A, we classified SLN containing micrometastases or metastases (≥0.2mm), which according to the TNM classification correspond to the tumor-positive lymph nodes (pN1). With regard to their size and number of cells pro microscopic field in a $20^x$ enlargement (as explained in Table 5), the tumor clusters or cells with dimensions smaller than 0.2mm are classified under Group B, C and D.

**Table 5: Proposed extension of the TNM classification for lymph nodes**

| GROUP | CONDITION | ILLUSTRATION |
|---|---|---|
| **A** | Metastases or micrometastases (bigger than or equal to 0.2mm, as described by the AJCC (AJCC 2002b). |   |
| **B** | Tumor cell clusters* smaller than 0.2mm or more than 10 occult tumor cells** visible in a $20^x$ microscopic enlargement, in one or several microscopic fields. |   |
| **C** | Less than 10 occult tumor cells**, visible in a $20^x$ microscopic enlargement in one or several microscopic fields. |   |
| **D** | Single sporadic occult tumor cells**, visible in a $20^x$ microscopic enlargement in one or several microscopic fields. |   |
| **Negative** | No evidence of metastases, micrometastases, isolated or sporadic occult tumor cells. | / |

* Tumor clusters are lesions with dimensions <0.2mm that consist of a group of tumor cells placed together. They have clear histological evidence of malignant activity.

**Occult tumor cells are single tumor cells with dimensions <0.2mm.

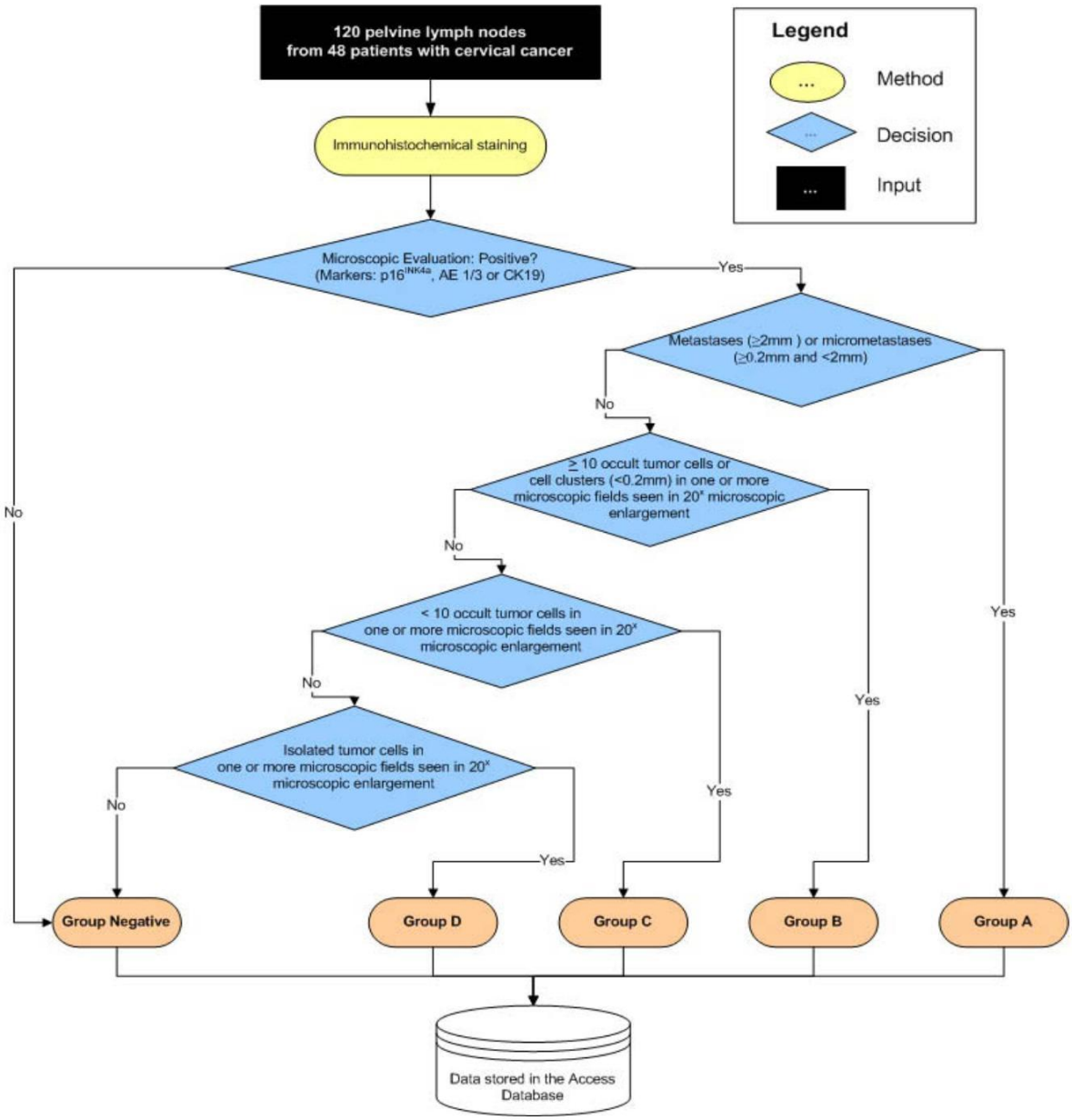



**Figure 31: Flow chart for microscopic evaluation of SLN after IHC staining**

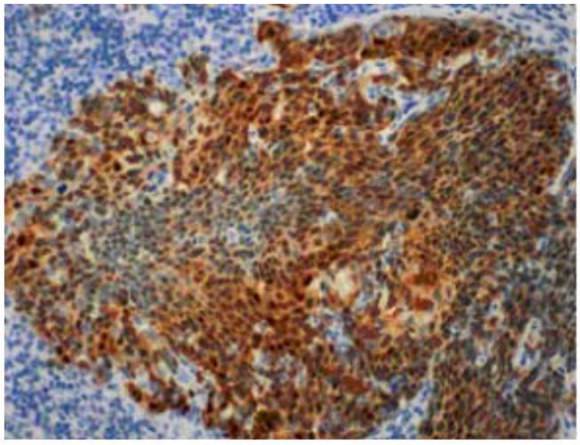
For each marker, we prepared one to two serial sections which were then immunohistochemically stained for $p16^{INK4a}$, CK19 and AE1/3. The evaluation of the SLN tissue sections for presence of tumor cells or clusters was done using a computerized light microscope. As explained in Table 5, our classification is based on the size and number of tumor entities evident in a microscopic enlargement $20^{x}$. Tumor positive lymph nodes were classified under Groups A, B, C and D. The conditions that had to be followed for the microscopic diagnosis are illustrated in the flow chart in Figure 31. Lymph nodes containing tumor

metastases or micrometastases which, due to their size, were easily distinguishable from the tumor-free tissue are classified under Group A. However, it was more complicated to classify SLN with "borderline" morphologic findings (Groups: B, C, D and Negative). During the microscopic evaluation for example, cases with presence of only a single isolated tumor cell (in a $20^x$ microscopic view) were identified and it was hard to determine whether it was a tumor cell or a staining artifact which means that the SLN could be positive (Group D) or negative (as explained and illustrated in Paragraph 4.2.1). In such situation, false positive or negative results are possible. To minimize the possible interpretation mistakes, all our microscopic findings are discussed with and confirmed by at least one expert pathologist.

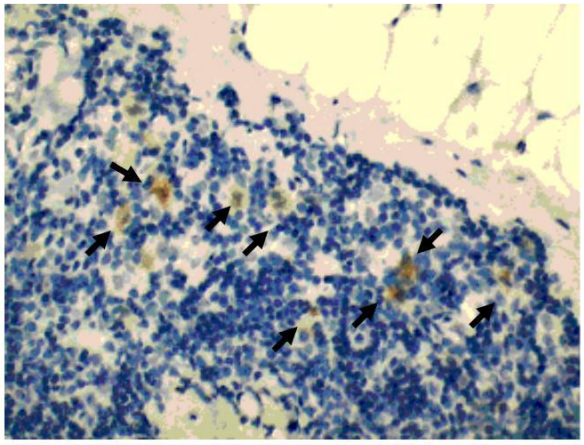
### 3.2.1 Staining for $p16^{INK4a}$

The cyclin dependent kinase inhibitor $p16^{INK4a}$ is a tumor suppressor gene that is overexpressed in several cancer entities associated with HPV infection such as cervical cancer and melanoma (DakoCytomation 2010, Mihic-Probst et al. 2006). In the dysplastic cervical epithelium cells, $p16^{INK4a}$ is overexpressed in cytoplasm and nuclei. The overexpression of $p16^{INK4a}$ indicates an active expression of the viral oncogene E7. The division cycle of somatic cells is regulated by a family of protein kinases known as "cyclin dependent kinases". In cervical squamous carcinomas, the functional inactivation of pRB by the HPV oncoprotein E7 (as explained Paragraph 1.1.3) leads towards an overexpression of $p16^{INK4a}$ in the cell nuclei and cytoplasm which selects $p16^{INK4a}$ as a surrogate marker for diagnosing several primary cancers associated with a HPV infection. A statistically significant correlation between the $p16^{INK4a}$ overexpression and the LN status was found in patients with primary melanomas. In this case, a strong overexpression of $p16^{INK4a}$ in the primary tumor correlated with a $p16^{INK4a}$ overexpression in migrating tumor cells to lymph nodes (Mihic-Probst et al. 2006).

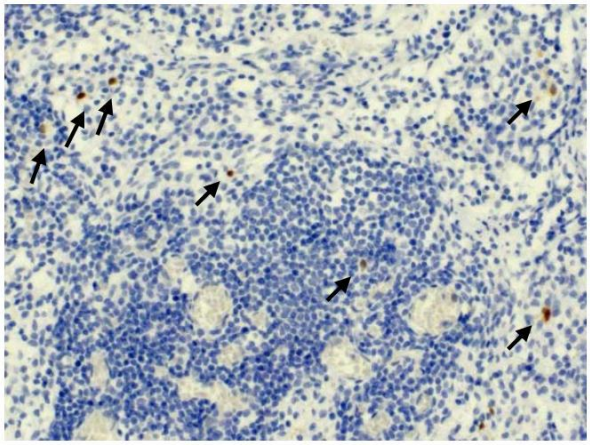
a) Group A: Enlargement 20x



b) Group B: Enlargement 20x



c) Group C: Enlargement 20x



d) Group D: Enlargement 40x

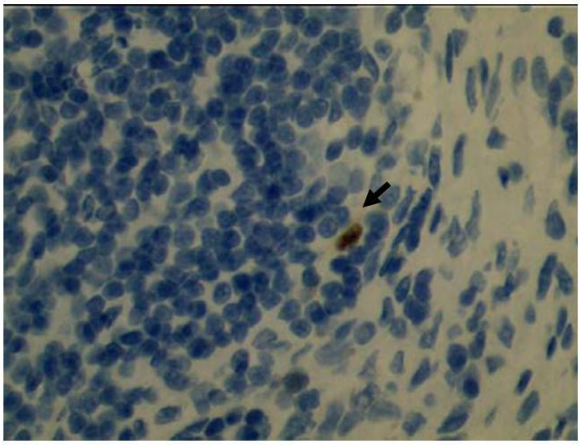


**Figure 32: Illustration of the staining for p16$^{INK4a}$ for Groups A, B, C and D**

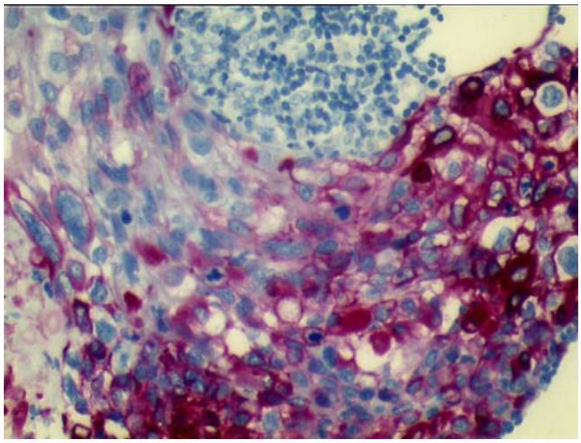
In this study, p16$^{INK4a}$ was selected to be validated as a potential maker for SLN of women with primary cervical cancer and positive HPV16. The 120 SLN were microscopically evaluated for tumor presence. Figure 32 illustrates the p16$^{INK4a}$ immunostaining in lymph nodes classified as positive for Groups A, B, C and D. p16$^{INK4a}$ expression stained tumor cells in brown. These cells became easily detectable due to the diffuse nuclear additionally to some cytoplasm staining. The brown-stained tumor cells were distinguishable from the blue surrounding lymphocyte cells (non-tumor cells).

### 3.2.2 Staining for CK19

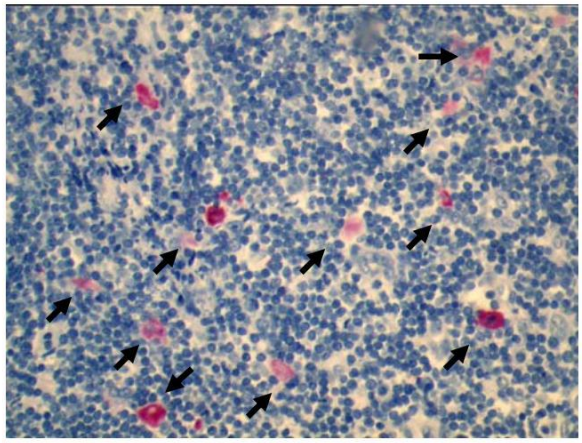
Human epithelial cell intermediate-sized filaments or cytokeratins are expressed in different epithelia and are nowadays used as markers for cell differentiation (Moll 1994, Moll et al. 1982). The subsets of cytokeratins that epithelial cells

express depend mainly on the type of epithelium, point of time of terminal differentiation and the stage of development (Paragraph 1.2.3.2.1). The cytokeratins' profile tends to remain constant when epithelium undergoes malignant transformation. This is the reason why cytokeratins were chosen as epithelial markers in tumor tissues. The cytokeratin 19 protein (CK19) is a widely distributed cytokeratin, being expressed in various epithelia, including many simple epithelia. CK19 is used to detect several epithelial tumors such as the cervical and breast cancer and tumors of lungs (Alexander-sefre et al. 2002, Benjamin 1995, Moll 1994). We proposed CK19 as a potential marker for LN of patients with primary epithelial CCa. When CK19 is expressed, the nucleus and cytoplasm of tumor cells are stained in pink color. Pictures of four SLN that were positive for CK19 under Groups A, B, C and D are provided in Figure 33.

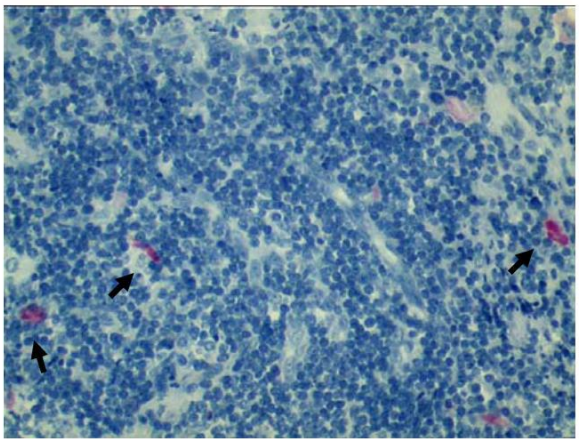
a) Group A: Enlargement $20^x$



b) Group B: Enlargement $20^x$



c) Group C: Enlargement $20^x$



d) Group D: Enlargement $20^x$

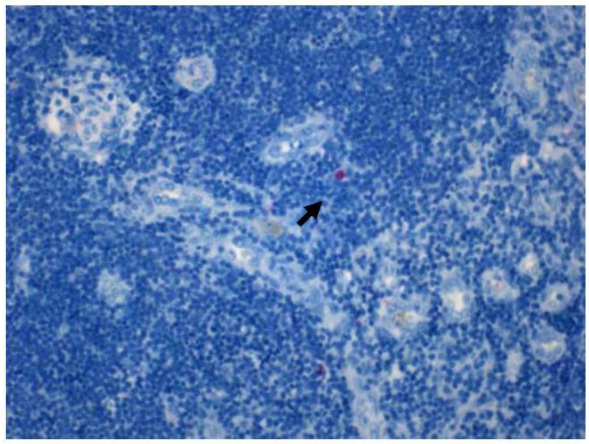
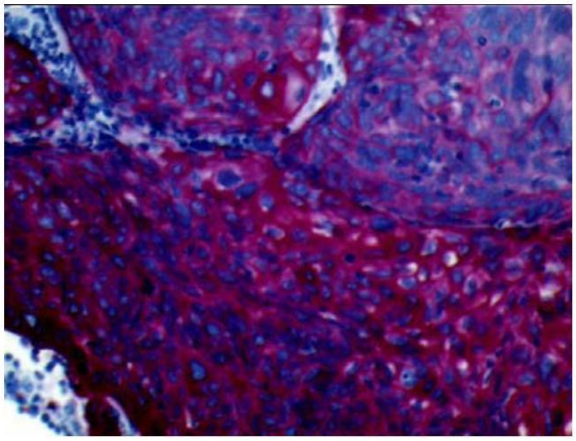


**Figure 33: Staining for the cytokeratin marker CK19 for Groups A, B, C, and D**

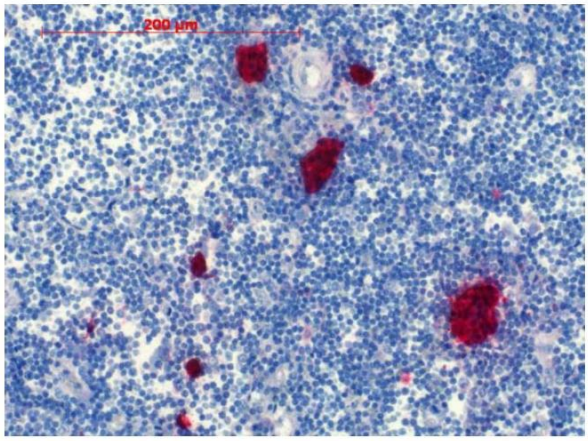
### 3.2.3 Staining for AE1/3

The cocktail cytokeratin marker AE1/3 is a pan cytokeratin antibody that is expressed in tumor cells and is, therefore, used as marker to detect micrometastases or occult tumor cells in lymph nodes of patients with primary carcinoma of pancreas (Kurahara et al. 2007), billiary tract carcinoma (Lara et al. 2003) and colorectal cancer (Nicastri et al. 2007) (Paragraph 1.2.3.2). AE1/3 could also be a reliable marker for LN of patients with primary cervical cancer. In this study, the expression of AE1/3 is used for the detection of lymph node tumor cells or clusters. AE1/3 stained LN tumor cells in pink color. Tumor cells were easily identifiable from the non-tumor cells. Not only micrometastases or occult tumor cells and tumor cell deposits classified under Groups A, B and C were clearly evaluated as positive, but also single isolated tumor cells (Group D) were easily distinguishable. Pictures taken at a $20^x$ microscopic enlargement from four different SLN that were positive for AE1/3 (Groups A, B, C and D) are illustrated in Figure 34.

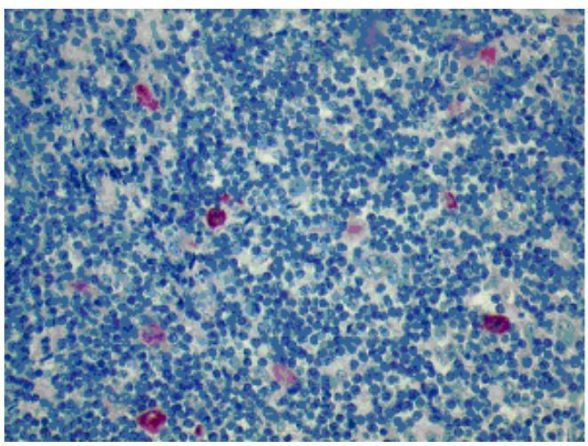
a) Group A: Enlargement $20^x$



b) Group B: Enlargement $20^x$




c) Group C: Enlargement $20^x$



d) Group D: Enlargement $20^x$

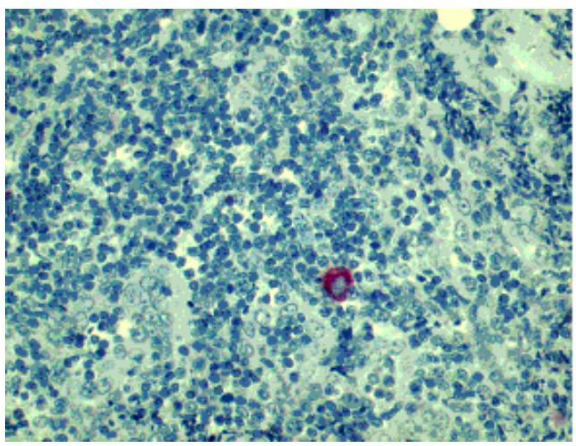


**Figure 34: Staining for AE1/3 (Groups A, B, C, and D)**

## 3.3 Renewed evaluation of pN0 lymph nodes

The probability for tumor detection increases when the LN are evaluated by both conventional histopathology (H-E staining) and additional tumor specific markers for LN (e.g. IHC markers). Conventional H-E staining can only detect tumor clusters; occult tumor cells cannot be identified. If LN tumors with longitudinal dimension smaller than micrometastases (<0.2mm) are responsible for the poor prognosis of women treated for their cervical cancer, then specific markers for lymph nodes are necessary to detect these residual tumors that cannot be detected by the routine H-E staining. In this study, the 120 SLN were initially examined for tumor presence by conventional histology. All SLN underwent a renewed serial sectioning and were stained for H-E and three potential immunohistochemical markers for lymph nodes ($p16^{INK4a}$, CK19 and AE1/3). The 85 SLN that were originally negative by histopathology (pN0) were microscopically re-evaluated for presence of tumor cells or clusters. For each marker and each single Group, the sensitivity, specificity and overall agreement were determined. The results, however, were discrepant and, thus, unsatisfying due to the blurred borders between the Groups B, C and D caused by multiple sectioning of SLN: A SLN could be classified to be in one Group based on consideration of one section and to be in another Group based on results of another section. For this reason, to reduce the inter-group discrepancies, we considered Group combinations AB, ABC and ABCD for determination of the statistical agreement between the IHC markers. Group AB includes all SLN that were found positive for any of the Groups A and B; Group ABC includes all positive SLN for any of the Groups A, B and C and Group ABCD includes all positive SLN for any of the Groups A, B, C and D.

Figure 35 summarizes the results of the re-evaluation of the 85 histologically negative SLN for each single Group A, B, C and D as well as Group combinations AB, ABC, and ABCD. After the re-sectioning, detection of numerous SLN containing tumor cells or clusters smaller than micrometastases (Groups B, C, and D) which could not be identified by the routine H-E staining was expected, but diagnosing micrometastases or metastases was absolutely unexpected. Surprisingly, metastases or micrometastases (Group A) were detected in 4 SLN (4.7%). As expected the number of detected tumor positive SLN was

considerable: For the Group ABCD, 57 SLN (67.1%) were positive for $p16^{INK4}$; only 33 SLN (38.8%) were positive for CK19 and 58 SLN (68.2%) were positive for AE1/3. Considerable discrepancies for the detection of isolated and sporadic tumor cells by the three markers were obvious due to the blurred borders between the single Groups caused by multiple sectioning. The extent of these discrepancies is assessed further in Paragraph 4.2.1.

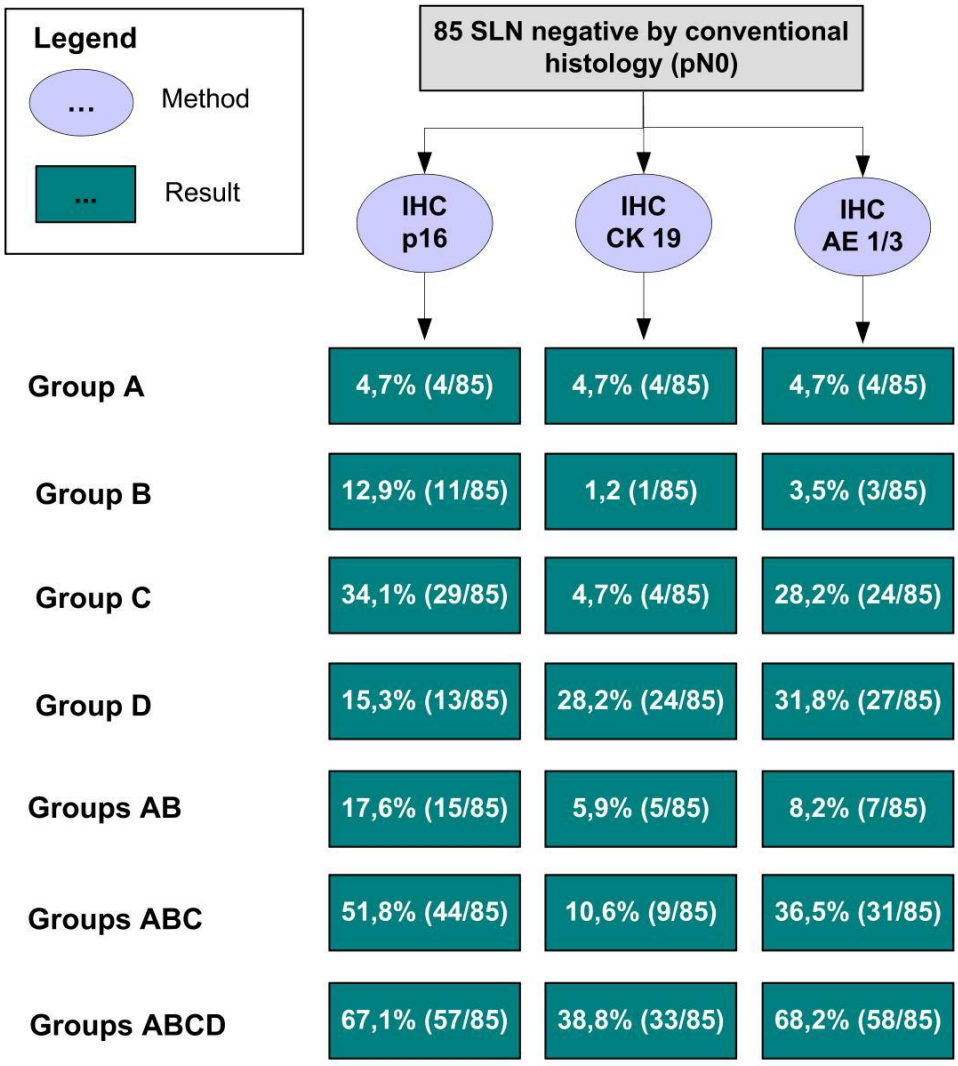



**Figure 35: Results of the IHC evaluation of the histologically negative SLN**

### 3.3.1 Statistical correlation between the IHC markers

We used the “two by two” Table of Agreement to evaluate the association between the four markers. By convention, the performance of a diagnostic test is

based on sensitivity, specificity and the overall agreement. Sensitivity (detection of true tumor positive lymph nodes) and specificity (detection of true tumor negative lymph nodes) are computed independently from the disease prevalence or the probability of positive LN in the entire population at a point of time. The validation of staining quality of the three IHC markers showed that the cocktail cytokeratin marker AE1/3 provided the best staining comparing to $p16^{INK4a}$ and CK19; therefore it was considered as Rater1 or our "gold standard". In this paragraph, the agreement between AE1/3 (alone or in combination with CK19) and the other IHC markers will be assessed. Subsequently, each of the IHC markers (AE1/3, $p16^{INK4a}$ and CK19) will also be compared to the HPV mRNA molecular marker.

As mentioned in Paragraph 3.3, the 85 SLN that were negative by the histopathology were reevaluated. The comparison between AE1/3 and CK19 is shown in Table 6. The ability to detect all true pN1 cases (Group A) was 100%, indicating that in all four SLN the micrometastases or metastases with dimensions larger than or equal to 0.2mm were detected by both markers AE1/3 and CK19. The agreement between the two markers was, however, worst for the detection of tumor cells or clusters smaller than 0.2mm. A number of false negative cases were found increasingly for Groups AB, ABC and ABCD. In these cases, CK19 might have failed to stain the small-sized tumor cells or clusters. The fact that CK19 often stained heterogeneously or failed to stain even micrometastases or metastases, explains its inadequate ability to detect a number of true tumor positive LN. Consequently, comparing to AE1/3, CK19 failed to stain a considerable number of occult tumor cells having an impact on the sensitivity which is quite low for the Group AB (57.14%), Group ABC (25.81%) and Group ABCD (48.28%). Sensitivity for Group ABCD was higher than the sensitivity for Group ABC. However, as long as Group D consists of single tumor cells that could be simply staining artifacts, a false positive or negative rate is possible for group D. Subsequently, the Group ABC seems to be most relevant for further considerations (Paragraph 4.1.3). In our study, for Group ABC, CK19 only detected 8 out of 31 SLN that were truly positive by AE1/3. As a result, the specificity and, consequently, the overall agreement for Group ABC were much lower than for Groups A and AB.

**Table 6: Agreement between AE1/3 and CK19 (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) AE1/3 (Group A)

| CK19 | Positive | Negative | |
|---|---|---|---|
| **Positive** | True (+) 4 | False (+) 0 | 4 |
| **Negative** | False (-) 0 | True (-) 81 | 81 |
| **Total** | 4 | 81 | 85 |

Sensitivity: 100%
Specificity: 100%
Overall agreement: 100%

b) AE1/3 (Group AB)

| CK19 | Total | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 4 | False (+) 1 | 5 |
| **Negative** | False (-) 3 | True (-) 77 | 80 |
| **Total** | 7 | 78 | 85 |

Sensitivity: 57.14%
Specificity: 98.72%
Overall agreement: 95.29%

c) AE1/3 (Group ABC)

| CK19 | Positive | Negative | |
|---|---|---|---|
| **Positive** | True (+) 8 | False (+) 1 | 9 |
| **Negative** | False (-) 23 | True (-) 53 | 76 |
| **Total** | 31 | 54 | 85 |

Sensitivity: 25.81%
Specificity: 98.15%
Overall agreement: 71.76%

d) AE1/3 (Group ABCD)

| CK19 | Total | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 28 | False (+) 5 | 33 |
| **Negative** | False (-) 30 | True (-) 22 | 52 |
| **Total** | 58 | 27 | 85 |

Sensitivity: 48,28%
Specificity: 81.48%
Overall agreement: 58.82%

Being aware that HR-HPV are present in almost all primary cervical cancers and that the HR-HPV surrogate marker $p16^{INK4a}$ demonstrates increased immune staining in worsening grades of CIN; we proposed $p16^{INK4a}$ as a potential marker for detecting migrating tumors in lymph nodes of patients with primary CCa. The agreement between AE1/3 and $p16^{INK4a}$ (considering AE1/3 as the "gold standard") is shown in Table 7. The evaluation of the 85 histologically negative SLN by means of the "two by two" Table of Agreement showed a perfect 100% agreement between the two markers for Group A. However, this agreement decreased for Groups AB, ABC and ABCD whereas the worst agreement was for Group ABC. A number of tumor cells were only detected by AE1/3 resulting in an increasing number of SLN that were false negative by $p16^{INK4a}$ for Group AB, ABC and ABCD. Due to their small size, it is possible that single tumor cells were only present in one of the serial sections of SLN tissue. As a consequence of the altering number of tumor cells seen in a $20^{x}$ microscopic view, after serial sectioning, the same SLN was often categorized under different Groups of our classification (B, C or D). Accordingly, discrepancies in the agreement between

AE1/3 and $p16^{INK4a}$ are obvious. An increasing number of true tumor positive SLN is detected by AE1/3 but there is also a number of cases that were only positive by $p16^{INK4a}$ (false positive SLN). The potential reasons for this situation are explained in Paragraph 4.1.2.

**Table 7: Agreement between AE1/3 and $p16^{INK4a}$ (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) AE1/3 (Group A)

| p16 | Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 4 | False (+) 0 | 4 |
| **Negative** | False (-) 0 | True (-) 81 | 81 |
| **Total** | 4 | 81 | 85 |

Sensitivity: 100%
Specificity: 100%
Overall agreement: 100%

b) AE1/3 (Group AB)

| p16 | Total Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 5 | False (+) 10 | 15 |
| **Negative** | False (-) 2 | True (-) 68 | 70 |
| **Total** | 7 | 78 | 85 |

Sensitivity: 71.43%
Specificity: 87.18%
Overall agreement: 85.88%

c) AE1/3 (Group ABC)

| p16 | Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 22 | False (+) 22 | 44 |
| **Negative** | False (-) 9 | True (-) 32 | 41 |
| **Total** | 31 | 54 | 85 |

Sensitivity: 70.79%
Specificity: 59.26%
Overall agreement: 63.53%

d) AE1/3 (Group ABCD)

| p16 | Total Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 45 | False (+) 12 | 57 |
| **Negative** | False (-) 13 | True (-) 15 | 28 |
| **Total** | 58 | 27 | 85 |

Sensitivity: 77.59%
Specificity: 55.56%
Overall agreement: 70.59%

By combining the two cytokeratin markers CK19 and AE1/3 together to stain two serial sections of the same SLN, more tumor cells or clusters were detected in SLN biopsies comparing to the use of a single cytokeratin marker.

We consider the combination of two cytokeratin markers to identify the SLN as positive if at least one of the markers identifies the tissue as positive. Let us assume that CK19 identified a serial section of a lymph node as negative and AE1/3 identified another section of the same SLN as positive. In such situation we consider the SLN as positive. The only case when the SLN is considered to be negative is when both markers identify it as negative. This behavior corresponds to the definition of the logical operator "*or*" (Enderton 2001): "*x or y*" is considered to be true if either "x" is true or "y" is true or both "x" and "y" are

true. The expression "*x and y*" is only true if both "x" and "y" are true but not if only one of them is true. Obviously, the use of the logical operator "*and*" is not appropriate in our case since we would miss to identify SLN as positive if only one of the markers identifies it as positive and another marker identifies it as negative. A corresponding truth table for the logical disjunction "CK19 *or* AE1/3" as well as logical conjunction "CK19 *and* AE1/3" is provided in Table 8. In the following, the notation "CK19 *or* AE1/3" is used to denote that a combination of markers CK19 and AE1/3 is used.

**Table 8: Truth table for the combination of CK markers (operators „*and*" and „*or*")**

| CK19 | AE1/3 | "CK19 *and* AE1/3" | "CK19 *or* AE1/3" |
|---|---|---|---|
| positive | positive | positive | positive |
| positive | negative | negative | positive |
| negative | positive | negative | positive |
| negative | negative | negative | negative |

By using the "two by two" Table of Agreement we calculated the percentage at which the two cytokeratin markers agreed with $p16^{INK4a}$. Statistical results showed that the combination of two cytokeratins agreed better with $p16^{INK4a}$ (overall agreement for Group ABCD: 76.47%) than AE1/3 alone (overall agreement for Group ABCD: 70.59%).

However, the percentage of detected true positive or true negative SLN and the overall agreement between the markers remained unsatisfying. The best sensitivity to detect tumor cell clusters or single tumor cells was for Group ABCD (79.3%) and the best specificity was for Group AB (87%) (Table 9). The agreement between the two cytokeratins together and $p16^{INK4a}$ is, however, similar to the agreement between AE1/3 alone and $p16^{INK4a}$. Respectively, the overall agreement was 63.5% and 64.7% for the Group ABC which includes the SLN that are truly positive (Table 7, Table 9). These statistical results show that the evaluation of lymph nodes by two cytokeratin markers together ("AE1/3 *or* CK19") might offer comparatively better results than using only one of these two markers but it is more expensive, time consuming and without major benefits. Therefore, staining by AE1/3 alone would be a better alternative.

**Table 9: Agreement between "AE1/3 *or* CK19" and p16$^{INK4a}$ (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a)

AE1/3 *or* CK19 (Group A)

| p16 | Positive | Negative | Total |
|---|---|---|---|
| **Positive** | True (+) 4 | False (+) 0 | 4 |
| **Negative** | False (-) 0 | True (-) 81 | 81 |
| **Total** | 4 | 81 | 85 |

Sensitivity: 100%
Specificity: 100%
Overall agreement: 100%

b)

AE1/3 *or* CK19 (Group AB)

| p16 | Positive | Negative | Total |
|---|---|---|---|
| **Positive** | True (+) 5 | False (+) 10 | 15 |
| **Negative** | False (-) 3 | True (-) 67 | 70 |
| **Total** | 8 | 77 | 85 |

Sensitivity: 62.50%
Specificity: 87.01%
Overall agreement: 84.71%

c)

AE1/3 *or* CK19 (Group ABC)

| p16 | Positive | Negative | Total |
|---|---|---|---|
| **Positive** | True (+) 23 | False (+) 21 | 44 |
| **Negative** | False (-) 9 | True (-) 32 | 41 |
| **Total** | 32 | 53 | 85 |

Sensitivity: 71.88%
Specificity: 60.38%
Overall agreement: 64.71%

d)

AE1/3 *or* CK19 (Group ABCD)

| p16 | Positive | Negative | Total |
|---|---|---|---|
| **Positive** | True (+) 50 | False (+) 7 | 57 |
| **Negative** | False (-) 13 | True (-) 15 | 28 |
| **Total** | 63 | 22 | 85 |

Sensitivity: 79.37%
Specificity: 68.18%
Overall agreement: 76.47%

### 3.3.2 Comparison of marker gene expression at the RNA and protein level

HPV mRNA is a diagnostic marker for cervical cancer that was present in all primary cervical carcinomas of women that were enrolled in this study. It could be a valid marker for detecting migrating malignant cells in lymph nodes of patients with HR-HPV positive CCa. In this section we will statistically measure how good the three selected IHC markers (p16$^{INK4a}$, CK19, AE1/3) agree with the molecular marker HPV mRNA. According to our findings concerning the presence of tumor cells or clusters in 85 histologically negative SLN, the sensitivity, specificity, and overall agreement are computed.

The RT-PCR analysis provides information about the presence or absence of HPV mRNA. For this reason we only know if the lymph node is HPV mRNA "positive" or "negative", while for each IHC marker the positive values are classified under Groups A, B, C and D. Therefore, the statistical correlation

between the IHC markers and HPV mRNA makes sense only for Group ABCD which includes all positive SLN by IHC. We have, therefore, calculated only the sensitivity for the Groups A, AB, ABC and ABCD and the overall agreement for Group ABCD only (Table 10, Table 11 and Table 12). The best sensitivity was for Group A (75%). However, only 3 out of 4 SLN that were positive under Group A by IHC were positive by HPV mRNA as well. The SLN that was positive by IHC but negative by HPV mRNA had a small cluster micrometastasis about 0.2mm located in the SLN margin. Perhaps, the micrometastasis was only present in the part of the SLN used for the IHC evaluation and, for this reason, was only detected by the IHC markers. Furthermore, HPV mRNA was positive in 42.86% of SLN that were positive by AE1/3 for Group AB (3/7 SLN), 45.16% for Group ABC (14/31 SLN) and 34.48% for Group ABCD (20/58 SLN). Not only the ability to detect true positive SLN, but also the ability to detect true negative SLN were not good for group ABCD (Sensitivity: 34.48%, Specificity: 74.07%). As a consequence, the overall agreement between AE1/3 and HPV mRNA for Group ABCD was only 47.06% (Table 10).

**Table 10: Agreement between AE1/3 and HPV mRNA (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) AE1/3 (Group A)

| | | **Positive** | | **Negative** |
|---|---|---|---|---|
| HPV mRNA | **Positive** | True (+) 3 | False (+) - | - |
| | **Negative** | False (-) 1 | True (-) - | - |
| | **Total** | 4 | - | - |

**Sensitivity: 75%**

b) AE1/3 (Group AB)

| | **Total** | **Positive** | | **Negative** |
|---|---|---|---|---|
| HPV mRNA | **Positive** | True (+) 3 | False (+) - | - |
| | **Negative** | False (-) 4 | True (-) - | - |
| | **Total** | 7 | - | - |

**Sensitivity: 42.86%**

c) AE1/3 (Group ABC)

| | | **Positive** | | **Negative** |
|---|---|---|---|---|
| HPV mRNA | **Positive** | True (+) 14 | False (+) - | - |
| | **Negative** | False (-) 17 | True (-) - | - |
| | **Total** | 31 | - | - |

**Sensitivity: 45.16%**

d) AE1/3 (Group ABCD)

| | **Total** | **Positive** | | **Negative** |
|---|---|---|---|---|
| HPV mRNA | **Positive** | True (+) 20 | False (+) 7 | 27 |
| | **Negative** | False (-) 38 | True (-) 20 | 58 |
| | **Total** | 58 | 27 | 85 |

**Sensitivity: 34.48%**

**Specificity: 74.07%**

**Overall agreement: 47.06%**

Using the “two by two” Table Statistics, the agreement of CK19 and HPV mRNA is shown in Table 11. HPV mRNA detected 75% of SLN that were positive by CK19 for Group A (3/4 SLN), 60% that were positive for Group AB (3/5 SLN), 44.44% for Group ABC (4/9 SLN) and 39.39% for Group ABCD (13/33 SLN). The results for Group A are identical to the results of the association of AE1/3 and HPV mRNA. Among all SLN that were positive for CK19 (Group ABCD), the ability of HPV mRNA to detect true positive cases was 39.39% (13/33 SLN were positive by both CK19 and HPV mRNA); the ability to detect true negative SLN was 73.08% (38/52 SLN were negative by both CK19 and HPV mRNA) and the overall agreement was 60% (Table 11).

**Table 11: Agreement between CK19 and HPV mRNA (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) CK19 (Group A)

| | | Positive | Negative |
|---|---|---|---|
| HPV mRNA **Positive** | True (+) 3 | False (+) - | - |
| HPV mRNA **Negative** | False (-) 1 | True (-) - | - |
| **Total** | 4 | - | - |

**Sensitivity: 75.00%**

b) CK19 (Group AB)

| | Total | Positive | Negative |
|---|---|---|---|
| HPV mRNA **Positive** | True (+) 3 | False (+) - | - |
| HPV mRNA **Negative** | False (-) 2 | True (-) - | - |
| **Total** | 5 | - | - |

**Sensitivity: 60.00%**

c) CK19 (Group ABC)

| | Positive | | Negative |
|---|---|---|---|
| HPV mRNA **Positive** | True (+) 4 | False (+) - | - |
| HPV mRNA **Negative** | False (-) 5 | True (-) - | - |
| **Total** | 9 | - | - |

**Sensitivity: 44.44%**

d) CK19 (Group ABCD)

| Total | | Positive | Negative | To |
|---|---|---|---|---|
| HPV mRNA **Positive** | True (+) 13 | False (+) 14 | 27 | |
| HPV mRNA **Negative** | False (-) 20 | True (-) 38 | 58 | |
| **Total** | 33 | 52 | 85 | |

**Sensitivity: 39.39%**

**Specificity: 73.08%**

**Overall agreement: 60.00%**

$p16^{INK4a}$, the surrogate marker for HPV infection, was expected to correlate with HPV mRNA better than the epithelial markers AE1/3 and CK19 (Table 12). On the contrary, the agreement between $p16^{INK4a}$ and HPV mRNA was also inadequate. For Group A, same to the correlation of HPV mRNA with CK19 and AE1/3, HPV mRNA detected 75% of SLN that were positive by $p16^{INK4a}$ (3/4 SLN). Furthermore, HPV mRNA was positive in 66.67% of SLN that were positive by $p16^{IN4a}$ for Group AB. The sensitivity decreased evidently for Group ABC

(36.36%) because HPV mRNA was detected only in 16 of the 44 SLN that were positive by $p16^{INK4a}$. Disappointing were also the results for Group ABCD where the sensitivity was only 40.35%, specificity was 85.71% and overall agreement 55.29%. Although, HPV mRNA detected more SLN that were true positive for $p16^{INK4a}$ than for "AE1/3 *or* CK19"; it also missed to detect a considerable number of positive SLN for $p16^{INK4a}$. HPV mRNA was negative in more SLN that were positive for $p16^{INK4a}$ than "AE1/3 *or* CK19" (Group B). Furthermore, for Group ABC, HPV mRNA showed lower ability to detect true positive SLN (sensitivity: 36.36%) than AE1/3 (Sensitivity: 45.16%).

**Table 12: Agreement between $p16^{INK4a}$ and HPV mRNA (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) $p16^{INK4a}$ (Group A)

| HPV mRNA | Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 3 | False (+) - | - |
| **Negative** | False (-) 1 | True (-) - | - |
| **Total** | 4 | - | - |

**Sensitivity: 75.00%**

b) $p16^{INK4a}$ (Group AB)

| HPV mRNA | Total | Positive | | Negative |
|---|---|---|---|---|
| **Positive** | True (+) 5 | False (+) - | - | |
| **Negative** | False (-) 10 | True (-) - | - | |
| **Total** | 15 | - | - | |

**Sensitivity: 66.67%**

c) $p16^{INK4a}$ (Group ABC)

| HPV mRNA | Positive | | Negative |
|---|---|---|---|
| **Positive** | True (+) 16 | False (+) - | - |
| **Negative** | False (-) 28 | True (-) - | - |
| **Total** | 44 | - | - |

**Sensitivity: 36.36%**

d) $p16^{INK4a}$ (Group ABCD)

| HPV mRNA | Total | Positive | | Negative |
|---|---|---|---|---|
| **Positive** | True (+) 23 | False (+) 4 | 27 | |
| **Negative** | False (-) 34 | True (-) 24 | 58 | |
| **Total** | 57 | 28 | 85 | |

**Sensitivity: 40.35%**

**Specificity: 85.71%**

**Overall agreement: 55.29%**

The combination of the two cytokeratin markers ("CK19 or AE1/3") did not agree much better with HPV mRNA than each cytokeratin alone (Table 13). This confirms again that the usage of two cytokeratin markers instead of the AE1/3 alone is not necessary. For Group ABCD, the overall agreement between AE1/3 alone and HPV mRNA (47.06%) was similar to the overall agreement between the two cytokeratins and HPV mRNA (48.24%). HPV mRNA was present in only 37.50% of SLN that were positive for the two cytokeratins for Group AB, in

43.75% of SLN for Group ABC and in 36.51% of SLN for Group ABCD. Although the specificity for Group ABCD increased to 81.82%, the overall agreement of "AE1/3 *or* CK19" and HPV mRNA was only 48.24%.

**Table 13: Agreement between "AE1/3 *or* CK19" and HPV mRNA (Groups A, AB, ABC, ABCD) for the histologically negative SLN**

a) AE1/3 *or* CK19 (Group A)

| HPV mRNA | | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 3 | False (+) - | - |
| **Negative** | False (-) 1 | True (-) - | - |
| **Total** | 4 | - | - |

**Sensitivity: 75.00%**

b) AE1/3 *or* CK19 (Group AB)

| HPV mRNA | Total | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 3 | False (+) - | - |
| **Negative** | False (-) 5 | True (-) - | - |
| **Total** | 8 | - | - |

**Sensitivity: 37.50%**

c) AE1/3 *or* CK19 (Group ABC)

| HPV mRNA | | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 14 | False (+) - | - |
| **Negative** | False (-) 18 | True (-) - | - |
| **Total** | 32 | - | - |

**Sensitivity: 43.75%**

d) AE1/3 *or* CK19 (Group ABCD)

| HPV mRNA | Total | Positive | Negative |
|---|---|---|---|
| **Positive** | True (+) 23 | False (+) 4 | 27 |
| **Negative** | False (-) 40 | True (-) 18 | 58 |
| **Total** | 63 | 22 | 85 |

**Sensitivity: 36.51%**

**Specificity: 81.82%**

**Overall agreement: 48.24%**

### 3.3.3 Statistical agreement by using the Kappa statistics

In this study, we used the Cohen's Kappa statistics to reconfirm the results obtained by the "two by two" Table of Agreement. The results of the agreement between the IHC markers computed using the Kappa statistics are summarized in Table 14. Also these results show that the agreement between the markers is "perfect" for Group A, confirming that all three markers are valid to detect micrometastases or metastases.

With regard to the staining quality, AE1/3 and $p16^{INK4a}$ seem to be potential markers for SLN. However, there was only a "fair agreement" between the "gold standard" AE1/3 and each of the two markers $p16^{INK4a}$ or CK19 for Group ABCD. According to Cohen's Kappa, although CK19 stained heterogeneously or failed to

stain numerous tumor cells, it achieved a “fair agreement” with both AE1/3 and $p16^{INK4a}$.

In addition to that, the combination of two cytokeratins CK19 and AE1/3 offered the best agreement with $p16^{INK4a}$; yet this agreement was only “moderate”. These results could have, however, been different if a larger sample size would be evaluated by this study.

**Table 14: Kappa and significance results for the agreement between IHC markers among histologically negative SLN**

| Agreement | Group | Kappa | Kappa interpretation |
|---|---|---|---|
| AE1/3 vs. p16 | A | 1.000 | Perfect agreement |
| | AB | 0.386 | Fair agreement |
| | ABC | 0.277 | Fair agreement |
| | **ABCD** | **0.328** | **Fair agreement** |
| AE1/3 vs. CK19 | A | 1.000 | Perfect agreement |
| | AB | 0.642 | Considerable agreement |
| | ABC | 0.282 | Fair agreement |
| | **ABCD** | **0.239** | **Fair agreement** |
| "AE1/3 *or* CK19" vs. p16 | A | 1.000 | Perfect agreement |
| | AB | 0.356 | Fair agreement |
| | ABC | 0.300 | Fair agreement |
| | **ABCD** | **0.437** | **Moderate agreement** |

The results of the Cohen’s Kappa agreement between the three IHC markers ($p16^{INK4a}$, CK19, AE1/3) and the molecular marker HPV mRNA are summarized in Table 15. In consideration of all positive SLN for IHC (Group ABCD), $p16^{INK4a}$ agreed with HPV mRNA better than the other IHC markers (“fair agreement”). The agreement between AE1/3, CK19 or the combination of both cytokeratins (“AE1/3 *or* CK19”) and the HPV mRNA was only “minor”. The explanation of the unsatisfying agreement could be the presence of the tumor cells in the region of the SLN tissue used for one method only (IHC or RT-PCR). Due to their small size, occult tumor cells or clusters might locate only in the part the SLN evaluated by one method.

**Table 15: Kappa results for agreement between IHC markers and HPV mRNA among histologically negative SLN**

| Agreement | Group | Kappa | Kappa interpretation |
|---|---|---|---|
| AE1/3 vs. HPV mRNA | ABCD | 0.065 | Minor agreement |
| CK19 vs. HPV mRNA | | 0.129 | Minor agreement |
| "AE1/3 *or* CK19" vs. HPV mRNA | | 0.120 | Minor agreement |
| p16 vs. HPV mRNA | | 0.205 | Fair agreement |

# Chapter 4: Discussion

In this chapter, the statistical results of the study are addressed and the reasons of the attained conclusions are discussed. According to the statistical outcomes, the validity on the four proposed markers for SLN is critically discussed in Paragraph 4.1.1. To address the reliability on the selected IHC markers ($p16^{INK4a}$, CK19, AE1/3) and the molecular marker (HPV mRNA), we judged the staining quality for the IHC markers and the ability of the four markers to detect tumor cells and clusters in lymph nodes. The prognostic role of small-size tumors smaller than micrometastases (<0.2mm) will be critically discussed. The impact of study limitations on the outcomes is discussed in Paragraph 4.2. In addition, compulsory research needed to be carried out by future studies is recommended in Paragraph 4.3.

## 4.1 Evaluation of markers reliability

The staining quality of the IHC markers ($p16^{INK4a}$, CK19 and AE1/3) is important for differentiating the tumor cells and clusters from the rest of LN tissue. When $p16^{INK4a}$ is overexpressed, the tumor cells are stained in brown color, whereas after expression of CK19 and AE1/3 the tumor cells are stained in pink.

Paragraph 4.1.1 discusses the results of the microscopic validation of the staining pattern based on pN1 SLN. The Paragraphs 4.1.2 and 4.1.3 evaluate subsequently the potential reasons of the unsatisfying agreement between the IHC markers and the molecular marker HPV mRNA with regard to tumor detection.

### 4.1.1 Validation of the staining quality of IHC markers

The staining quality for the three IHC markers AE1/3, CK19 and $p16^{INK4a}$ was microscopically validated as illustrated in Paragraph 3.1. For each of the three IHC markers, two serial-sections were prepared from each of the 35 available pN1 SLN and stained for the corresponding marker. To validate the staining quality of each marker, these two sections were microscopically compared between each other. Subsequently, for each SLN the sections stained for all markers were microscopically compared with each other. In both cases it was expected to obtain the same results when evaluating the sections of the same SLN.

However, while the markers AE1/3 and $p16^{INK4a}$ perfectly agreed with each other for the detection of micrometastases and metastases, CK19 failed to stain in one case, thus, classifying the SLN as “false negative”. Although the corresponding tumor cluster was easily identifiable due to the morphology of tumor cells and the positive results for $p16^{INK4a}$, AE1/3 and HPV mRNA (see Paragraph 3.1.2) the tissue was not stained for CK19.

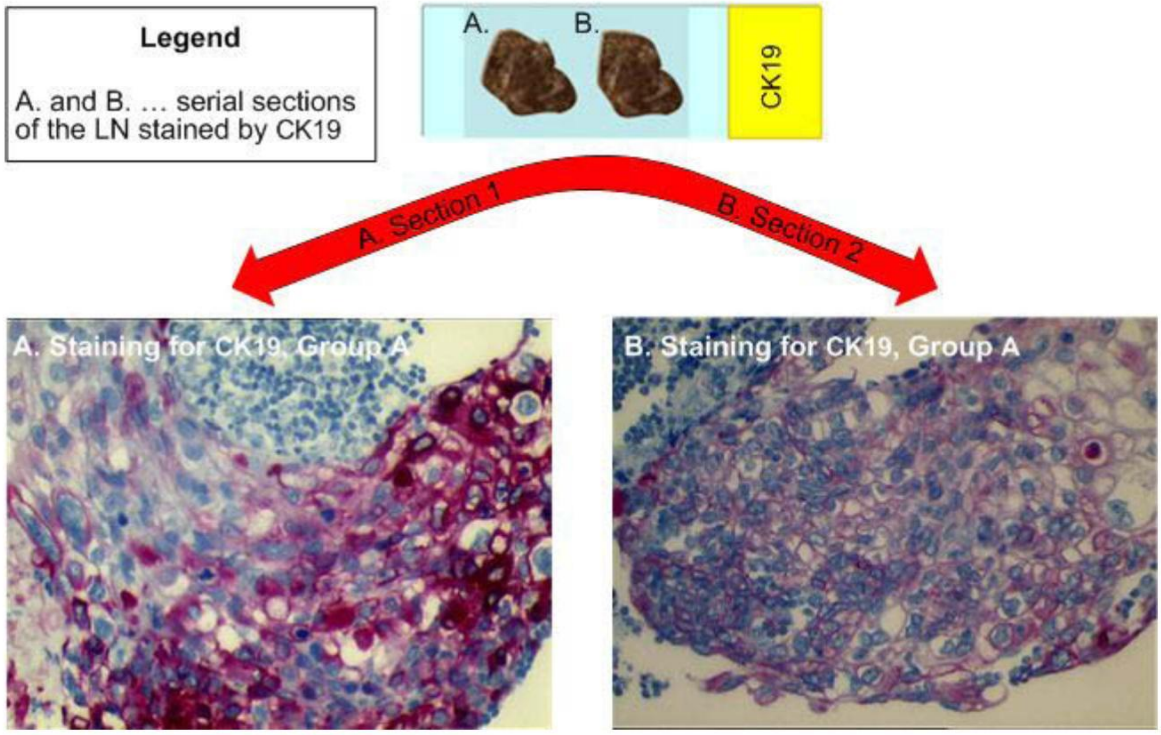



**Figure 36: Different staining intensities for CK19 in 2 serial sections of the same LN**

Furthermore, we noticed that staining for CK19 was heterogeneous in several SLN. Figure 36 provides pictures from two serial sections of one SLN. Both sections are stained for CK19. While comparing two serial sections of the same metastasis, a heterogeneous (Figure 36 A) and a weak staining (Figure 36 B) are obvious. Due to this finding, more discrepancies are expected for the detection of occult tumor cells (Groups B, C, and D) using CK19.

### 4.1.2 Agreement between IHC markers

As mentioned in Paragraph 4.1.1, in some cases CK19 provided a heterogeneous staining of tumor clusters (micrometastases or metastases) or failed to stain at all. The reliability of CK19 is, therefore, particularly questionable for the detection of occult tumor cells or clusters with dimensions smaller than 0.2mm (classified under Group B, C or D). Consequently, a number of false negative results can be expected as certain cells could fail to be stained and a number of SLN is expected to be incorrectly classified as negative.

Apparently, after a renewed serial sectioning, only 63 of 120 evaluated SLN were positive for CK19, while 90 SLN were positive for $p16^{INK4a}$ and 91 for AE1/3 (Group ABCD). Out of these positive SLN, sporadic or isolated tumor cells or clusters smaller than micrometastases (Group B, C and D) were identified in 29 SLN stained for CK19, 53 SLN stained for $p16^{INK4a}$ and 54 SLN stained for AE1/3 (Appendix 1). These numbers show obvious discrepancies between staining for CK19 and the other two IHC markers (AE1/3 and $p16^{INK4a}$) which confirm the unreliability on CK19 and indicate that CK19 fails to identify a considerable number of positive LN for occult tumor cells.

In this study, the “two by two” Table of Agreement was used to statistically measure the agreement between the selected potential markers for lymph nodes ($p16^{INK4a}$, AE1/3, CK19 and HPV mRNA). Validation of the staining quality of the three IHC markers suggested AE1/3 as the best marker. AE1/3 stains tumor clusters homogeneously and has the highest potential to detect tumor cells. For this reason AE1/3 was chosen as our “gold standard” and was compared to the other two markers $p16^{INK4a}$ and CK19. The results of the statistical agreement are summarized in Table 16. There was a “perfect agreement” between the three

markers for the detection of cluster micrometastases and metastases classified under Group A. All cluster tumors larger than or equal to 0.2mm that were detected by AE1/3 were also identified by $p16^{INK4a}$ and CK19. Therefore, the sensitivity, specificity and, consequently, the overall agreement for Group A were 100%.

Discrepancies were, however, obvious for the detection of small-size tumors classified under Groups B, C and D. Not all tumor cells or tumor clusters smaller than micrometastases (<0.2mm) that were detected by AE1/3 were also detected by the other markers. Therefore, the overall agreement was unsatisfying for Group B, C and D. The best overall agreement was achieved for Group AB when comparing AE1/3 with CK19. For Group ABC the best agreement was again between AE1/3 and CK19 and for Group ABCD between the two cytokeratins AE1/3 and CK19 together and $p16^{INK4a}$ (Table 16). Although CK19 detected more true negative cases than $p16^{INK4a}$, it cannot be suggested as a reliable marker for the lymph nodes knowing that CK19 often stains heterogeneously and fails to stain present tumors.

**Table 16: Summarized statistical agreement between IHC markers (computed by "two by two" Table of Agreement)**

| | **Sensitivity (in %)** | | | | **Specificity (in %)** | | | | **Overall Agreement (in %)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A** | **AB** | **ABC** | **ABCD** | **A** | **AB** | **ABC** | **ABCD** | **A** | **AB** | **ABC** | **ABCD** |
| **AE1/3 vs. CK19** | 100 | 57.1 | 25.8 | 48.3 | 100 | 98.7 | 98.2 | 81.48 | **100** | **95.3** | **71.8** | 58.8 |
| **AE1/3 vs. p16** | 100 | 71.4 | 70.8 | 77.6 | 100 | 87.2 | 59.3 | 55.6 | **100** | 85.9 | 63.5 | 70.6 |
| **"AE1/3 *or* CK19" vs. p16** | 100 | 62.5 | 71.9 | 79.4 | 100 | 87 | 60.4 | 68.2 | **100** | 84.7 | 64.7 | **76.5** |

On the other hand, $p16^{INK4a}$ showed a considerable number of false positive cases for Groups AB, ABC and ABCD as well, causing the drop of specificity. During the microscopic evaluation of the pelvic lymph nodes of a patient diagnosed with infection we have noticed an obvious overexpression of $p16^{INK4a}$ (Figure 37). This patient had no evidence of a primary or secondary tumor which was re-confirmed by experts at the Institute of Pathology, University Clinic Jena. Though, several cells were stained in brown by $p16^{INK4a}$. According to our classification, the LN would be categorized under Group B as more than 20 stained cells were counted in a $20^{x}$ microscopic enlargement. Based on this case, we assume that $p16^{INK4a}$ might be overexpressed in non-tumor cells of lymph

nodes and could, therefore, provide a number of false positive cases especially for Groups B, C and D. In fact, our statistical results confirm that $p16^{INK4a}$ offered the highest number of false positive SLN for the Group ABC and, consequently, reached the worst agreement with AE1/3. If $p16^{INK4a}$ were falsely overexpressed in LN and the LN were, consequently, classified as positive, these patients would undergo an overtreatment which might increase morbidity without any survival benefit. The number of false positive stained SLN for $p16^{INK4a}$ could explain the decreasing specificity for Groups AB, ABC and ABCD. Further research should test the potential overexpression of $p16^{INK4a}$ in LN of patients that do not suffer any malignant disease. If the overexpression of $p16^{INK4a}$ will be evident in disease-free LN; then $p16^{INK4a}$ should be excluded as a potential marker for migrating tumor cells in lymph nodes.

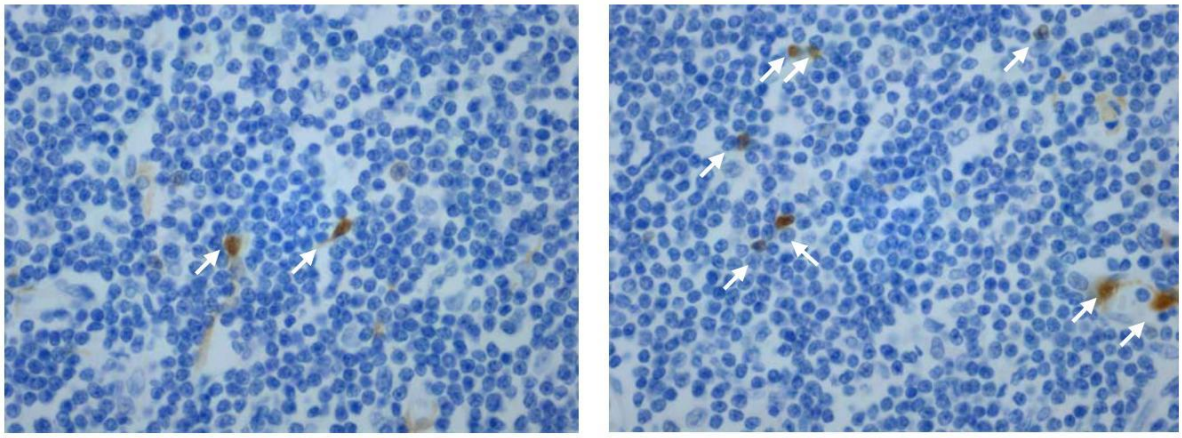


**Figure 37: Staining for $p16^{INK4a}$ of the LN of a patient with no CCa ($20^{x}$ enlargement)**

### 4.1.3 Agreement between IHC and HPV mRNA markers

In Paragraph 3.3.2, we compared our selected IHC markers with the HPV molecular marker (HPV mRNA). To statistically measure the association between these markers by using the “two by two” Table of Agreement, each of the IHC markers was considered as “gold standard“ successively.

While there are two valid states for the HPV mRNA (present/positive or absent/negative), the positive result of an IHC marker is further divided into four Groups (A, B, C, and D). To determine the agreement between IHC markers taken as “gold standard” and HPV mRNA we, however, abstained from

considering the single positive subgroups of the IHC markers and calculated the agreement between HPV mRNA and IHC for the Group ABCD (comprising all positive SLN for any of the groups A, B, C or D) only. Table 17 summarizes the outcomes of the correlation between $p16^{INK4a}$, CK19, AE1/3 and HPV mRNA. Evidently, for Group ABCD, the agreement between the markers did not result to be satisfying. A reason of the inadequate statistical agreement might be the sample lymph node tissue. Each SLN tissue was divided into two parts: One part was used for the immunohistochemistry and the other one for the RT-PCR procedure, meaning that the two methods evaluated different regions of the SLN. However, the poor results do not doubt the reliability of the methods used; both methods are highly sensitive but due to their small size the occult tumor cells can be occasionally located just in one part of the SLN and, thus, be detectable only by one of the methods (see also Paragraph 4.2.2).

**Table 17: Statistical agreement between IHC markers and HPV mRNA (computed by "two by two" Table of Agreement)**

| | **Sensitivity (in %)** | | | | **Overall Agreement (in %)** |
|---|---|---|---|---|---|
| | **A** | **AB** | **ABC** | **ABCD** | **ABCD** |
| **AE1/3 vs. HPV mRNA** | 75 | 42.86 | 45.16 | 34.48 | 47.06 |
| **CK19 vs. HPV mRNA** | 75 | 60 | 44.44 | 39.39 | 60 |
| **p16 vs. HPV mRNA** | 75 | 66.67 | 36.36 | 40.35 | 55.29 |
| **"AE1/3 *or* CK19" vs. HPV mRNA** | 75 | 37.5 | 43.75 | 36.51 | 48.24 |

It was expected that the HPV surrogate marker $p16^{INK4a}$ agrees with the molecular marker HPV mRNA better than the other two IHC markers (AE1/3 and CK19). Surprisingly, the overall agreement for Group ABCD was only 55.29%. Furthermore, comparing to the other two IHC markers, correlation of $p16^{INK4a}$ and HPV mRNA achieved the highest sensitivity for Group AB (Table 17). This suggests that apart of micrometastases and metastases (Group A) tumors classified under Group B have the highest probability to be detected by both methods (PCR and IHC) since the tumor cells have high chances to be distributed throughout the tissue. There were, however, ten SLN with tumor presence only in the part of the SLN used for IHC (Table 18). Thus, all the ten SLN were positive for $p16^{INK4a}$ (Group B) and negative for HPV mRNA. They were also positive for AE1/3 and sometimes for CK19 as well.

**Table 18: SLN negative by histology and HPV but positive for $p16^{INK4a}$ (Group B)**

| p16 (Group B) | | | |
|---|---|---|---|
| | **Histological number** | **AE 1/3** | **CK 19** |
| 1. | 16177/03 | Group D | Negative |
| 2. | 30836/03 | Group A | Group A |
| 3. | 11432/03 | Group D | Negative |
| 4. | 9397/03 IX | Group C | Negative |
| 5. | 16438/03 III | Group C | Negative |
| 6. | 10569/02 III | Group D | Group D |
| 7. | 29524/03 IX | Group B | Group D |
| 8. | 23301/03 | Group C | Negative |
| 9. | 17891/01 | Group C | Group C |
| 10. | 13014/03 | Group C | Negative |

When comparing each of the IHC markers with the HPV mRNA, the best overall agreement of 60% was, although not expected, between CK19 and HPV mRNA for Group ABCD (Table 17). As mentioned in Paragraph 4.1.1, it has been observed that in some cases CK19 provides a heterogeneous staining of micrometastases or metastases or fails to stain them at all. For this reason it is expected that CK19 would often fail staining occult tumor cells or clusters smaller than 0.2mm (Groups B, C and D). According to the “two by two” Table of Agreement, the decision on how much two markers agree with each other is dependent on the number of true positive and true negative cases for the marker taken as “gold standard”. Generally, if a marker identifying less positive cases is chosen as “gold standard”, it delivers a good overall agreement even when compared to a better marker. Thus, when we measured the agreement between either $p16^{INK4a}$ or AE1/3 (respectively chosen as the “gold standard”) and CK19, it was observed that due to the fact that CK19 only detects a part of true positive occult tumor cells (identified by other markers) the sensitivity was very low. When comparing CK19 with HPV mRNA, except for Group A, the number of true positive SLN detected by CK19 was much lower than the true positive cases detected by $p16^{INK4a}$ and AE1/3 in a comparison with HPV mRNA. For Group ABCD, CK19 detected 33 positive SLN while $p16^{INK4a}$ detected 57 positive SLN and AE1/3 detected 58 positive SLN. The specificity, in contrary, was usually high since a number of lymph nodes that were positive by the other IHC markers (Groups B, C and D) or HPV mRNA were negative by CK19. This explains the better overall agreement between CK19 and HPV mRNA).

For Group ABCD, the correlation of AE1/3 and HPV mRNA has reached the worst agreement comparing to the two other IHC markers (Table 17). As explained and illustrated in the previous chapter, comparing to the other two IHC markers, AE1/3 provided the best staining quality. AE1/3 was, therefore, expected to detect occult tumor cells or clusters better than CK19 and similar to $p16^{INK4a}$.

Lymph nodes classified under Group B have higher chances that their tumor cells or clusters are distributed throughout the tissue. However, four SLN that were positive for AE1/3 (Group B) were negative for HPV mRNA (Table 19). None of these four SLN was negative for $p16^{INK4a}$ and only one was negative for CK19. This reconfirms that HPV mRNA was negative because the part of SLN tissue used for the PCR analysis was free of tumor cells or some tumor cells were present but non-active and, therefore, they could not be detected. Concluding, the low sensitivity, specificity and unsatisfying agreement between AE1/3 and HPV mRNA do not exclude AE1/3 as a potential marker for SLN.

**Table 19: Histological and HPV negative SLN that were positive by AE1/3 (Group B)**

| AE 1/3 (Group B) | | | |
|---|---|---|---|
| | **Histological number** | **CK 19** | **p16** |
| 1. | 9397/03 XII | Negative | Group C |
| 2. | 30836/03 | Group A | Group A |
| 3. | 29524/03 IX | Group D | Group B |
| 4. | 30487/02 IV | Group D | Group C |

We also measured the agreement between two cytokeratin markers together (CK19 or AE1/3) and HPV mRNA which was not better than the agreement of each IHC marker alone. This result suggests that usage of two cytokeratins for tumor detection in SLN is not necessary; it is time consuming, costly and with no major benefits.

Finally, although the agreements were unsatisfying, the results are, however, acceptable. The reasons for main discrepancies are:

- the inconsistent evaluation of the SLN tissue by both methods (each method evaluates a different region of the SLN tissue) (Paragraph 4.2.2)

- HPV mRNA is only able to detect metabolically active cells. If the tumor cells classified under Group B, C or D are not-active, HPV mRNA will be negative
- the agreement between a poor marker (e.g. CK19) taken as “gold standard” and a good marker (e.g. HPV mRNA) might incorrectly result to be good.

The molecular marker at the RNA level (HPV mRNA) provides an obvious advantage comparing to the IHC markers. Our results exclude CK19 as a potential marker for lymph nodes. Furthermore, larger studies with bigger sample size are needed to validate whether $p16^{INK4a}$, AE1/3 and HPV mRNA are adequate markers for LN.

## 4.2 Limitations of the study

The results of this study might have been influenced by several restrictions which are explained in the coming paragraphs:

- Limitations of the proposed classification (Paragraph 4.2.1): the proposed classification presents no clear-cut differentiation between Groups B, C and D which makes the interpretation of results difficult but also explains most discrepancies in the agreement between the IHC markers.
- For the IHC and RT-PCR analyses different parts of the LN tissue are used which is a reason for discrepancies in the agreement between IHC markers and HPV mRNA (Paragraph 4.2.2).
- Inadequate sample size (Paragraph 4.2.3): only 120 SLN were evaluated in this study. 85 out of them were negative by histology and re-assessed by IHC and PCR markers to validate the four proposed markers for lymph nodes.

### 4.2.1 Limitations of the proposed classification

In this study, to evaluate the lymph nodes of patients with primary cervical cancer we proposed a modification of the existing TNM classification. This classification

is meant to take into consideration not only micrometastases and metastases but also occult tumor cells or clusters with dimensions <0.2mm migrating to lymph nodes. The TNM classification is reliable for the detection of micrometastases and metastases (≥0.2mm). In the proposed classification, tumors ≥0.2mm are classified under Group A and are easily identifiable and clearly differentiable from the other positive Groups. However, classifying the distributed and isolated tumor cells or clusters smaller than 0.2mm is not easy as there is no strict differentiation between Groups B and C; C and D; D and Negative. Two serial sections were prepared and immunohistochemically stained by each IHC marker for about 80% of sentinel lymph nodes. We evaluated microscopically both sections and noticed that in numerous SLN one of the sections had more than 10 tumor cells in one or several microscopic views of $20^x$ enlargement classifying the SLN under Group B, but the second section had only less than 10 present tumor cells in one or several microscopic views of $20^x$ enlargement classifying the SLN under Group C (Figure 38). This means that there is no clear-cut to easily make a distinction between Groups B and C.

The same situation happened with Groups C and D. The heterogeneous distribution of tumor cells made it hard to differentiate Group C from D. Figure 39 illustrates a SLN where one of the two sections was classified under Group C and the second one under Group D. We have categorized such SLN under the highest positive Group, which means that in the case that Section 1 was Group B (Figure 38-A) and Section 2 was Group C (Figure 38-B), the SLN was classified under Group B. If one section of the same lymph node was classified as positive for Group C (Figure 39-A) and the second was positive for Group D (Figure 39-B) or Negative, then the SLN was classified under Group C.

There were also SLN with one positive section for Group D and the coming section negative. Such SLN were classified under Group D. Moreover, errors during IHC staining process (fixation) might happen and can cause artifacts in the histological preparation which makes the interpretation of results difficult. This situation is mostly possible for making the diagnosis of a sole isolated tumor cell found in a lymph node. Single tumor cells (Group D) might be simply artifacts of antibody cross-reactivity. Although the morphology of a tumor cell theoretically makes it easily detectable from the non-tumor cells; practically, in a number of

cases it was not easy to differentiate a tumor cell from a staining artifact even at a high microscopic enlargement. Both, tumor cells and artifacts are stained; therefore mistakes during classifying LN under Group D or Negative are possible. Due to the possible errors in evaluation of LN that are classified under Group D, it is reasonable to consider LN that are classified under Group ABC as surely positive while considering the Group D as unsure.

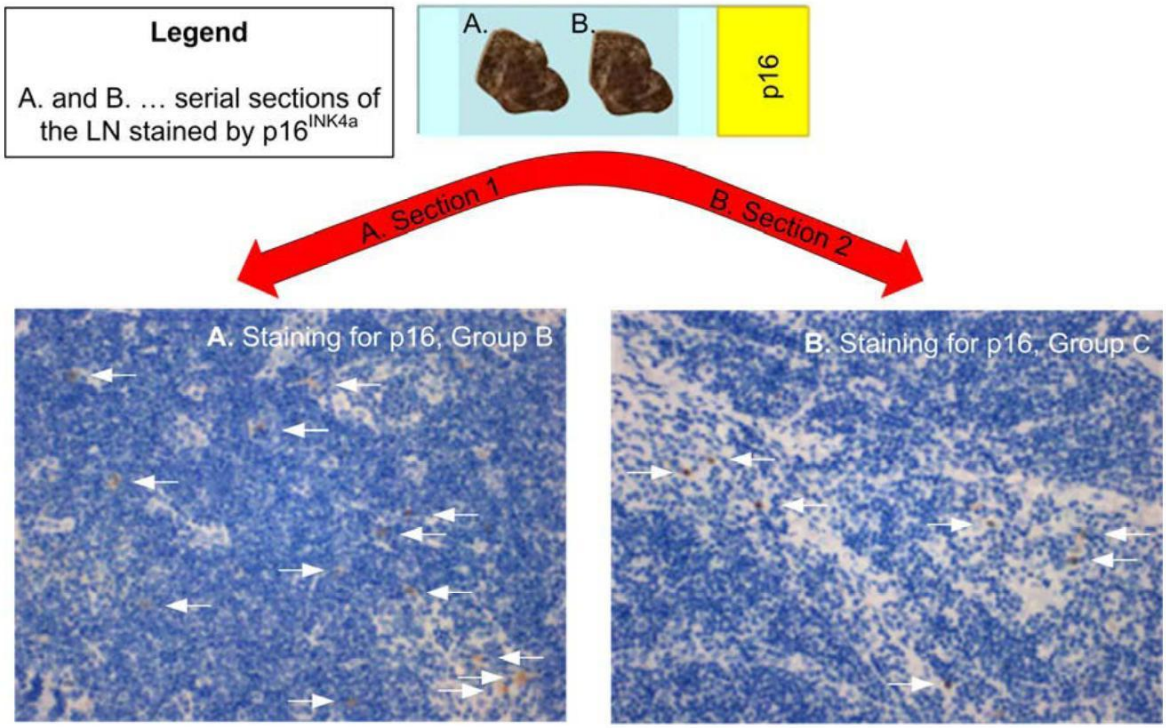



**Figure 38: Different Grouping (B and C) for two serial sections of the same SLN**

The above mentioned reasons show that there is no strict differentiation between Groups B and C, C and D, and D and Negative which shows that interpretation of results in such situations is highly subjective. This explains most divergences in statistical agreements between the IHC markers for detection of occult tumor cells and cell clusters <0.2mm. Obviously, all three IHC markers demonstrated major discrepancies for Groups B, C and D. In addition to that, it also implies that the tumor cells are irregularly distributed in the LN, suggesting that several serial interspersed sections of the LN tissue are necessary to be evaluated for tumor presence. Multiple intersperse sectioning is more accurate for the detection of occult tumor cells or cell clusters smaller than 0.2mm. The small size of occult tumor cells allowed the same LN to be classified under two different Groups which is however reasonable in cases where comparable number of tumor cells

is detected in both serial sections. If in the two sections extreme Groups are evident then the staining quality of the marker might be questionable. False-negative results occur when markers fail to stain, but also when the specimens are not completely representative of the lesion. The size of the LN tissue is also important. In several LN, a considerable part of the tissue consisted of fat and the tissue that could be examined was relatively small.

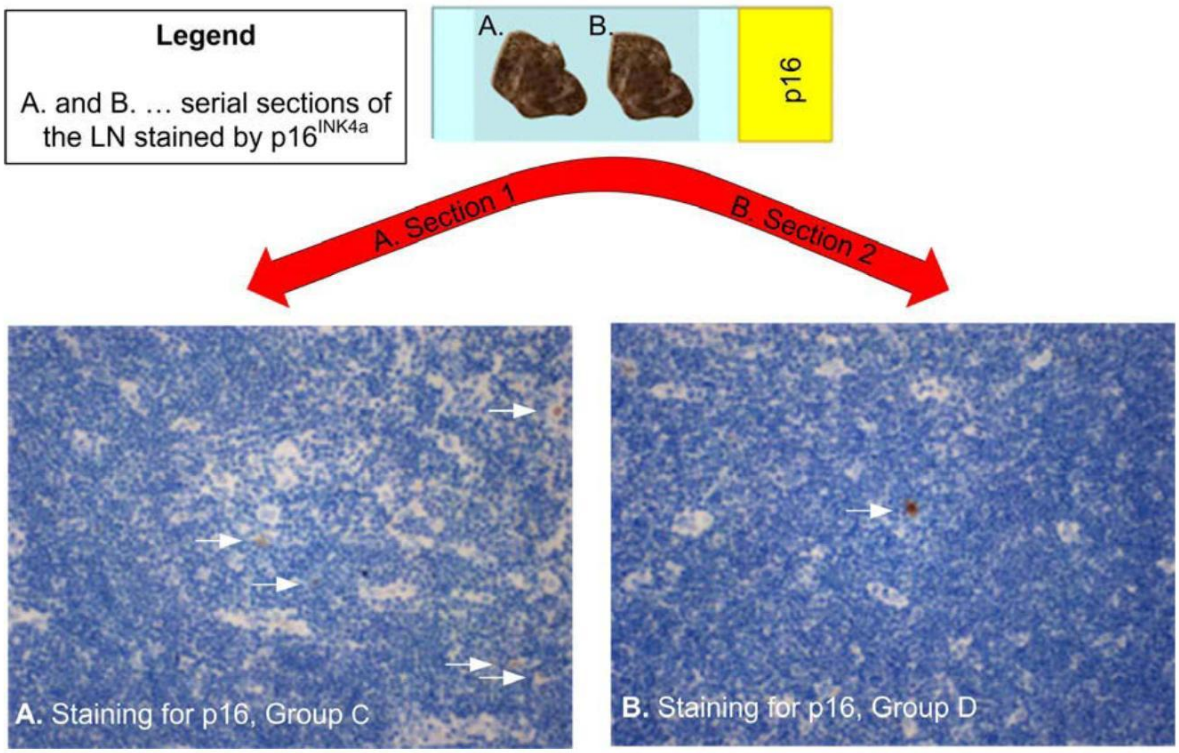



**Figure 39: Different Grouping (C and D) for two serial sections of the same SLN**

Although we listed some reasons that could explain the unsatisfying agreement between the IHC markers, according to the results of this study none of them can be selected as a valid marker for LN yet. The validity of the $p16^{INK4a}$, AE1/3 and HPV mRNA should, however, be researched further by larger studies.

### 4.2.2 Specimen evaluation by IHC and RT-PCR

The 120 SLN were initially sectioned and stained for H-E. The two serial sections that were performed were microscopically evaluated for present micrometastases and metastases which resulted that 35 SLN were pN1 and 85 were pN0.

Although pN0 status, the 85 SLN were re-sectioned and re-evaluated by IHC (with the help of the markers: H-E, $p16^{INK4a}$, CK19 and AE1/3) and PCR (selected marker: HPV mRNA). A part of the remaining tissue of each SLN was consequently used for the IHC and the other part for the PCR procedure. The diagnosis set by the first histopathological examination did not entirely correspond to the results after the renewed evaluation. After resectioning, out of the 35 histologically positive SLN, 4 SLN (11.43%) resulted negative by IHC (H-E, $p16^{INK4a}$ and AE1/3) (Appendix 1-c) and 5 SLN (14.28%) resulted negative by HPV mRNA (Appendix 1-a, 1-b). Furthermore, 4 out of the 85 histologically negative SLN resulted micrometastasis or metastasis positive (pN1) by all IHC markers (H-E, $p16^{INK4a}$, CK19 and AE1/3) (Appendix 1-c). In addition to that, a number of SLN resulted positive for occult tumor cells or cell clusters <0.2mm (classified under Groups B, C and D). However, IHC outcomes were not always corresponding to the PCR results (Appendix 1-a, 1-b). The main reason of these discrepancies is that some micrometastases and particularly occult tumor cells or tumor cell deposits could only be present in one SLN region and were, therefore, only detected by one method (RT-PCR or IHC). Consequently, an unsatisfying correlation occurred between the three IHC markers, but the correlation between IHC markers and HPV mRNA was much worse. Because of the small number of tumor cells and their small dimensions, the SLN that were positive either by IHC or RT-PCR could have had the tumor cells or clusters located only in the part of the tissue used for one method. Especially SLN classified under Group D have high chances to be classified as positive only by one method as isolated sole tumor cells might be located only in one region of the LN and the LN might result negative by other methods or markers. This does not mean that the unsatisfying results happened from the inaccuracy of the selected markers but the small number of evaluated SLN. This study, however, confirmed that a single marker shows poor sensitivity, whereas a combination of molecular and IHC tumor specific markers could offer the best sensitivity and specificity.

Furthermore, the selected diagnosing methods were not responsible for the inadequate results of the study. IHC is a sensitive method able to detect even single isolated tumor cells or tumor cell deposits. However, selection of tissue-specific markers remains of highest importance. On the other side, PCR method

is able to find a few copies of HPV mRNA in a single tumor cell. However, occult tumor cells or tumor cell clusters were often not detected by PCR (HPV mRNA was negative in numerous cases that were positive by IHC). Due to their small size, occult tumor cells and tumor cell clusters with dimensions smaller than micrometastases were often located only in one part of the LN tissue and, therefore, were identified only by one of the used methods. The IHC staining for $p16^{INK4a}$, CK19 and AE1/3 is performed in serial sections of the LN. If the RT-PCR used a section following the serial-sectioning done for the IHC, a better correlation between immunohistochemical markers and HPV mRNA would be possible.

### 4.2.3 Sample size of the LN tissue

A total of 48 women with primary CCa were enrolled in this study and 120 SLN from these women were evaluated for present occult tumor cells or tumor cell clusters. Only 85 SLN were histologically negative (pN0) and were re-sectioned to evaluate the reliability on the four potential markers for LN. Due to the unreliable staining quality for CK19 and the bad statistical results comparing to the other three markers, we excluded CK19 as a valid marker for lymph nodes. The reliability on the remaining markers ($p16^{INK4a}$, AE1/3 and HPV mRNA) was also not sufficient. The statistical agreements between the markers were influenced by the limited sample size. A larger study with involvement of more patients and more lymph nodes would certainly present more reliable statistical results. As example, after re-sectioning, 4 out of 85 SLN that were initially negative by histology resulted to be positive by IHC (H-E staining, $p16^{INK4a}$, CK19 and AE1/3). Because of the heterogeneous staining by CK19, a larger sample size might have an impact on the agreement between markers even for Group A. For this reason, validation of $p16^{INK4a}$, CK19 and AE1/3 as potential markers for lymph nodes remains to be determined by larger studies.

## 4.3 Questions raised for forthcoming studies

The following paragraphs contain discussions about

- the conventional method used to detect distant micrometastases or metastases in LN of patients with CCa
- the role of small-size occult tumor cells or clusters (<0.2mm) in the disease prognosis
- the prospective cancer management in the case that disseminated occult cancer cells or cell clusters are responsible for disease recurrence after the patients with CCa are treated.

### 4.3.1 Multi-sectioning IHC and PCR performed additionally to conventional histology

Conventional histopathological examination is a routine procedure used to detect metastases or micrometastases. This procedure, however, has several limitations regarding detection of occult tumor cells and clusters and the extent to which the tissue is evaluated. Moreover, micrometastases and tumor cell clusters are non-randomly distributed in LN and complicate the diagnosis when only a part of the tissue is examined.

Serial sectioning of a representative part of the LN has to provide the biggest possible longitudinal sections by cutting across the biggest part of the LN tissue to increase the chances for tumor detection. Several studies confirmed that the level of sectioning increases the probability to detect micrometastases (Noura et al. 2002). The frequency of micrometastases and metastases identification increases through multiple-sectioning of the lymph nodes (Kurahara et al. 2007, Lara et al. 2003, Noura et al. 2002). Lentz et al. imply that 15% of patients with early stage cervical carcinoma (FIGO stage: IA1, IB1, IB2) and pN0 LN have identifiable micrometastases in LN after a thorough immunohistochemical re-evaluation (Lentz et al. 2004). According to Juretzka et al., 8.1% of patients with primary CCa and pN0 lymph nodes had present micrometastases after reevaluation by IHC (Juretzka et al. 2004). If the conventional histology looked at the multiple sections in more than one part of the LN, an adequate part of the LN would be evaluated and, consequently, more micrometastases or metastases could be identified. Even in our study, among the 85 SLN that were negative by conventional histopathology (pN0) four SLN resulted positive (pN1) after a

renewed sectioning. Micrometastases or metastases were detected in the four SLN after re-sectioning and staining with H-E, $p16^{INK4a}$, CK19 and AE1/3. However, histopathologic examination of serial sections at more than one representative part of the lymph node is associated with higher costs and is time consuming. Furthermore, false negative results would still be possible.

In addition to the conventional histopathology, LN evaluation with the help of IHC or molecular markers increases chances for tumor detection because the two methods (IHC and PCR) can also detect the occult tumor cells or clusters <0.2mm. Our study showed that a single marker is, however, not specific enough for the reliable detection of occult tumor cells. A combination of tumor markers at the RNA and protein level might be the most reliable alternative to evaluate SLN of patients with cervical cancer. Molecular markers detected at the RNA level provide an obvious advantage (Fishta et al. 2007).

### 4.3.2 Prognostic study

To understand the reasons for the poor prognosis, all risk parameters including LN micrometastases, lymph-vascular space involvement, and surgical margins should be considered. Many clinico-pathological factors of cervical cancer are still controversial in their prognostic significance. Enough evidence talks about importance of lymph node status additionally to other clinico-pathological parameters such as tumor size, involvement of parametrical space, age and uterine body extension. Yuan et al. evaluated the prognostic significance of ten known clinico-pathological factors of 1.115 women with cervical cancer and found that for both recurrence and survival, pelvic LN metastases was the most critical factor. The findings of this study indicated that the LN status takes a more dominant role than a parametrical extension (Yuan et al. 1999).

Among patients with primary carcinoma of the cervix, disease recurrence is expected when the lymph nodes are involved (pN1). Patients with micrometastases-free LN (pN0) are expected to have satisfying disease survival. However, approximately 15% of patients with early-stage cervical cancer (FIGO stage IB) developed recurrence after being treated although their lymph nodes were pathologically free of micrometastases (pN0) (Delgado et al. 1990). Current opinions suggest that in patients with small cervical carcinoma, FIGO IB1 and

pN0 LN, tumor recurrence can be due to occult residual tumor cells that were not resected by surgery or that were disseminated to lymph nodes during surgical procedure and persisted in situ (Horn et al. 2005, Leys et al. 2000). These occult disseminated tumor cells in blood, LN and bone marrow escape the conventional tumor staging and might be responsible for the poor prognosis (Rack et al. 2002, Ross et al. 2004). The prognostic role of the neglected occult tumor cells and clusters smaller than 0.2mm in LN is still being researched. As explained in the previous paragraphs, the routine hematoxylin-eosin staining is efficient only for the detection of groups of tumor cells (e.g. micrometastases or metastases), but it fails to identify minimal amounts of disseminated tumor cells. The undetected or neglected migrating tumor cells or clusters that are smaller than micrometastases (Groups B, C, and D according to our classification) might influence the outcome of cervical cancer. Nowadays, it is still believed that the unknown benefits of treatment for these small lesions would not be more important than the morbidity caused by the treatment itself (Singletary et al. 2002, Singletary and Greene 2003).

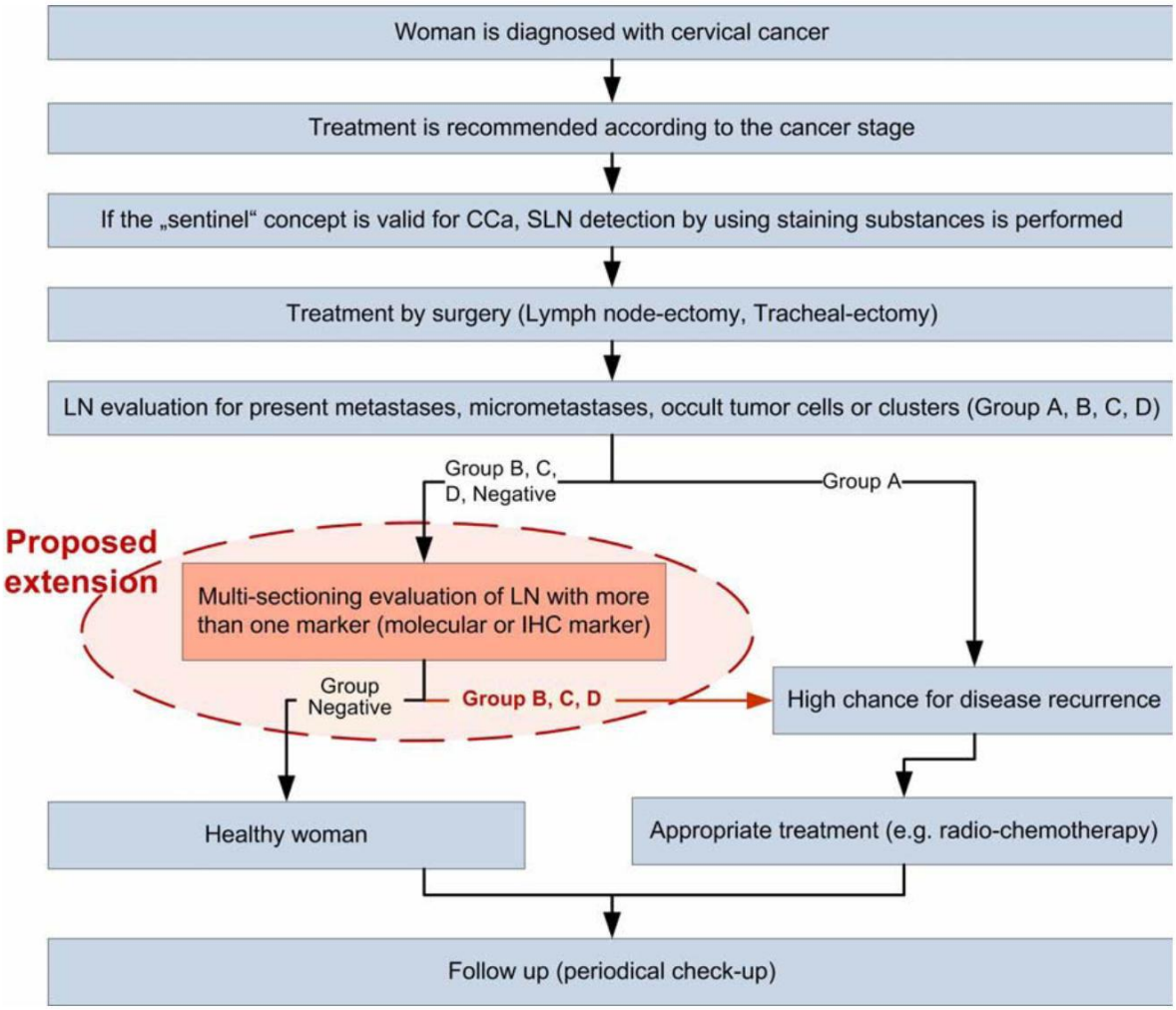



**Figure 40: Hypothetical prospective view of cervical cancer management**

In this study, out of the 85 histopathologically negative lymph nodes, 57 were positive for $p16^{INK4a}$, 33 for CK19, 58 for AE1/3 and 27 for HPV mRNA (Appendix 1-a). Although there were discrepancies among the tested tumor markers, it is obvious that a considerable number of lymph nodes, disregarding their pN0 status, contain tumor cells. This study enrolled a small number of patients; it would, however, be interesting to look over and compare the prognosis of these patients with positive LN for occult tumor cells or clusters smaller than 0.2mm (Groups B, C, and D) and the ones with LN free of tumor cells. Unfortunately, we had no information about the prognosis of the 48 women. However, a study with adequate sample size (number of patients) that would evaluate the prognosis of patients with pN0 status but with present occult tumor cells (that we classified under Group B, C and D) is needed to find out whether these tumor cells or clusters might have a prognostic meaning. If present tumor cells or clusters

smaller than 0.2mm in LN are the reason of the poor prognosis, then these would be the patients with highest expectation for disease recurrence or poor survival. Furthermore, valid tumor markers for LN could even replace the conventional H-E staining and CCa management protocols would need to be revised.

Enhanced methodology for detecting tumor presence in lymph nodes may provide the basis of improved post operative treatment (Barrera et al. 2003, Scheungraber et al. 2002) and may influence the patients' prognosis. In our study, the non-random distribution of tumor cells in lymph nodes required multiple sectioning to achieve high sensitivity. If prognostic studies confirm that patients with pN0 status but positive LN for occult tumor cells or clusters smaller than 0.2mm have a high risk for recurrence, then the CCa management protocol might need to be revised. Figure 40 illustrates a prospective view of cancer management, assuming that the presence of occult tumor cells or clusters smaller than 0.2mm in LN influences the prognosis of patients with primary CCa. Eventually, the role of these tumors (Groups B, C and D) in lymph nodes of patients with early stage CCa remains to be further investigated. As the prognostic relevance of occult tumor cells and clusters (<0.2mm) has not been proven yet, the significance of adjuvant therapy can be questioned for patients with otherwise good prognostic factors (Mirza et al. 2002, Tjan-Heijnen et al. 2001).

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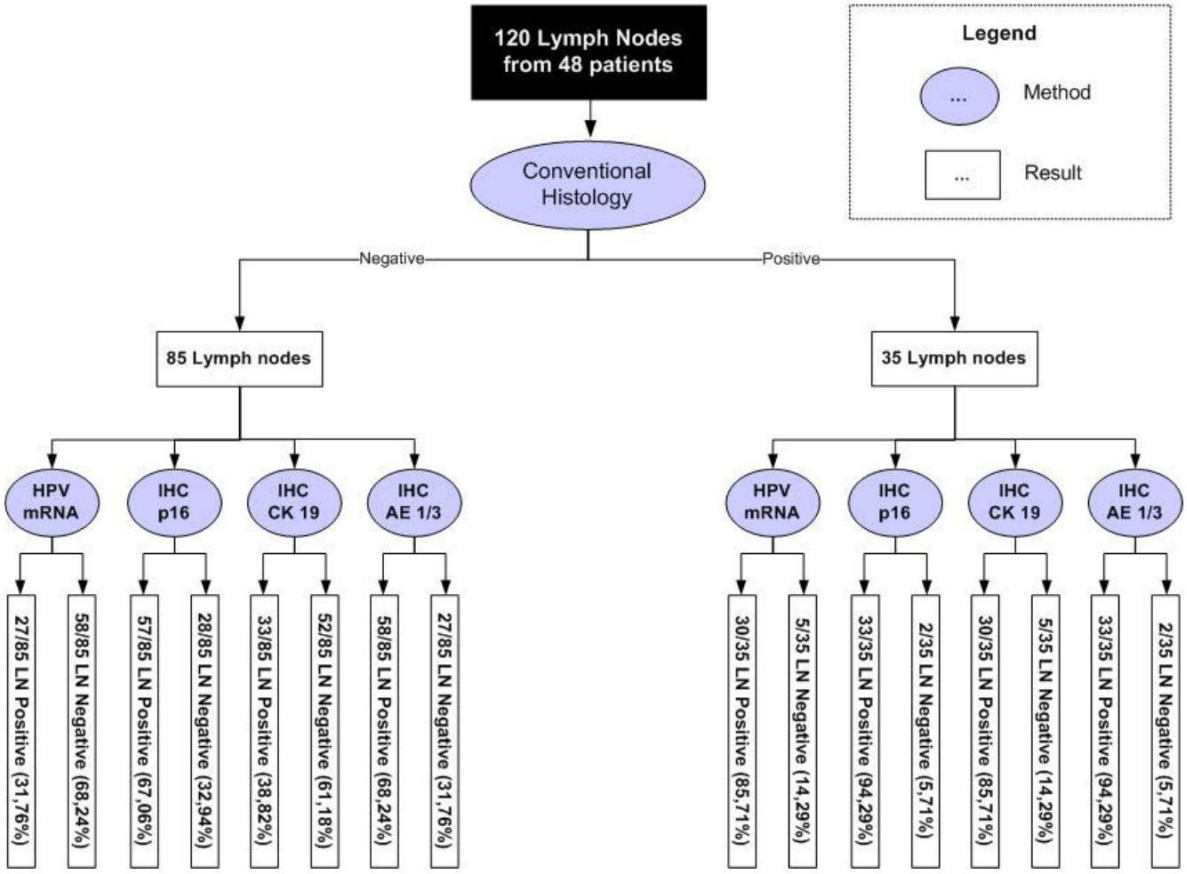
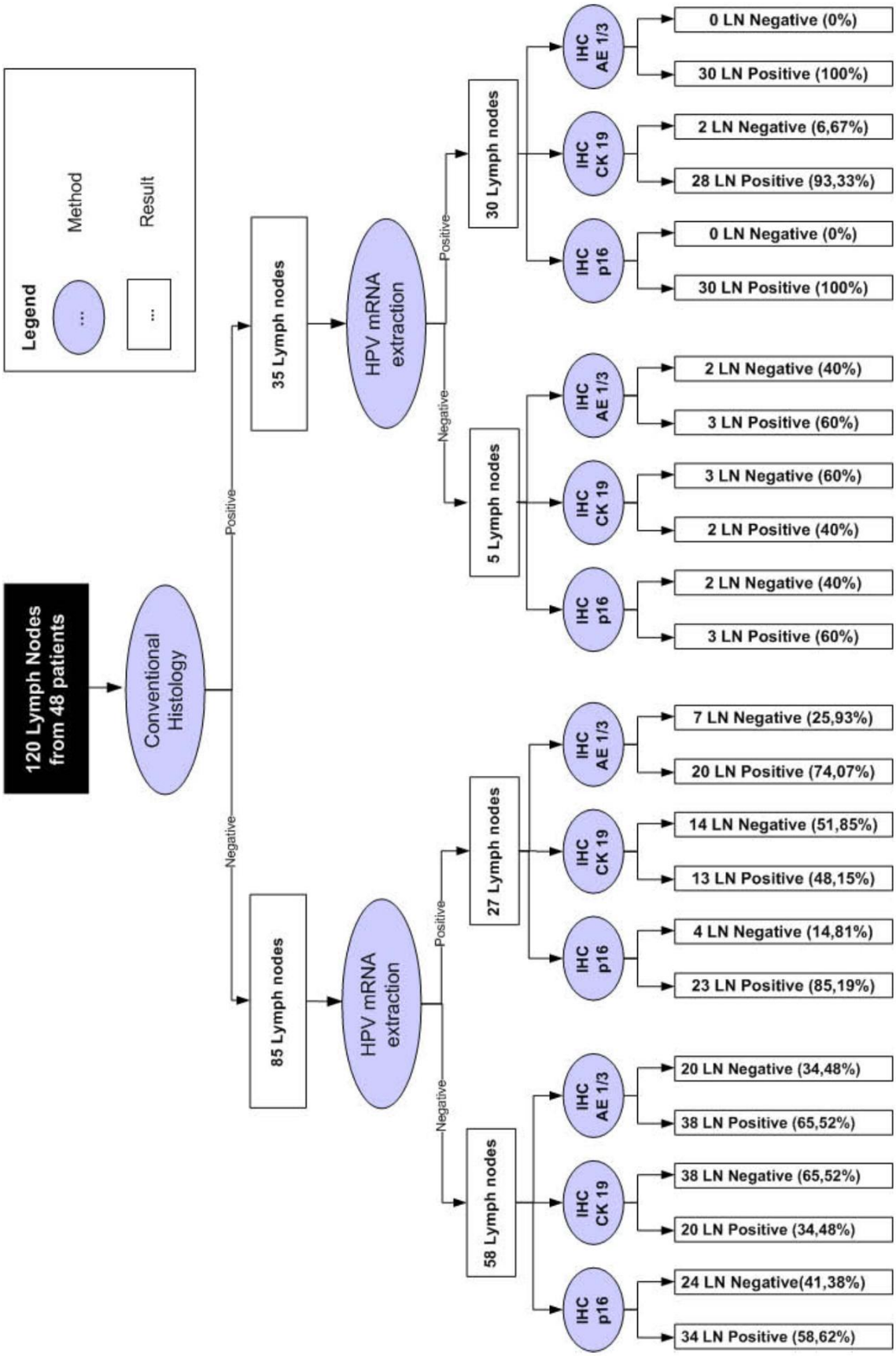
# Appendix 1: Descriptive results

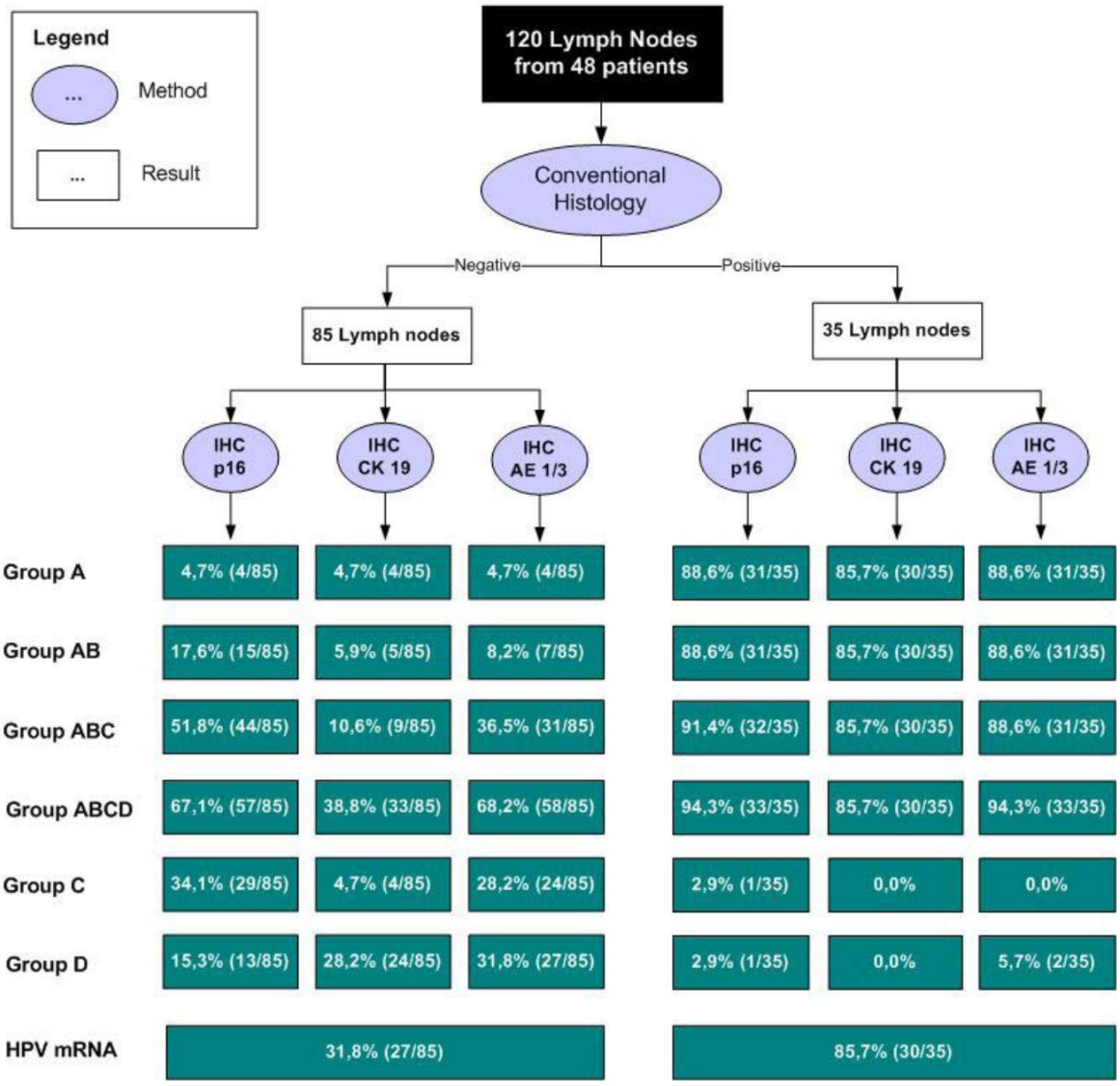
(a) SLN evaluated by the four selected markers (HPV mRNA, p16$^{INK4a}$, CK19 and AE1/3) depending on the conventional histology



(b) SLN evaluated by the three IHC markers ($p16^{INK4a}$, CK19 and AE1/3) depending on the results of conventional histology and the HPV mRNA



(c) SLN evaluated by the four selected markers (HPV mRNA, $p16^{INK4a}$, CK19 and AE1/3) depending on the results of conventional histology and classified under Group A, B, C, D or the combination in Group AB, ABC and ABCD for the results of immunohistochemistry



# Appendix 2: Data summary for each patient

| ***Patient Number*** | ***1*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **13014/03** | **VI** | B | Negative | C | H- | M- |
| **13014/03** | **II** | A | A | A | H+ | M- |
| **13014/03** | **V** | B | Negative | D | H- | M+ |
| **12752/03** | **ssI(1)** | A | A | A | H+ | M+ |
| **12752/03** | **ssI(2)** | A | A | A | H+ | M+ |
| **13014/03** | **III** | A | A | A | H+ | M+ |

| ***Patient Number*** | ***2*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **225/03** | **II 2.Ber** | A | A | A | H+ | M+ |
| **225/03** | **ssIII** | Negative | D | D | H- | M+ |
| **225/03** | **II** | A | A | A | H+ | M+ |

| ***Patient Number*** | ***3*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **16177/03** | **II** | B | Negative | D | H- | M- |
| **16086/03** | **ssIV** | C | D | D | H- | M- |
| **16086/03** | **ssIII** | Negative | Negative | Negative | H- | M- |
| **16086/03** | **ssII** | Negative | Negative | Negative | H- | M- |
| **16086/03** | **I** | Negative | D | D | H- | M- |
| **16177/03** | **III** | C | Negative | C | H- | M- |

| ***Patient Number*** | ***4*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **11432/03** | **II** | B | Negative | D | H- | M- |

| ***Patient Number*** | ***5*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **20788/02** | **VII** | Negative | Negative | Negative | H- | M- |

| ***Patient Number*** | ***6*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **23011/02** | **I** | D | Negative | Negative | H- | M- |

***Patient Number 7***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **23011/02** | **ss** | A | A | A | H+ | M+ |
| **23011/02** | **(1) 2.Ber** | A | A | A | H+ | M+ |
| **23011/02** | **(2) 2.Ber** | A | A | A | H+ | M+ |

***Patient Number 8***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **8212/03** | **VI** | A | A | A | H+ | M+ |
| **7997/03** | **ss** | A | A | A | H+ | M+ |
| **8212/03** | **V** | A | A | A | H+ | M+ |

***Patient Number 9***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **31082/02** | **VI** | A | A | A | H+ | M+ |
| **31048/02** | **ssIII** | A | A | A | H+ | M+ |
| **31082/02** | **VII** | A | A | A | H+ | M+ |

***Patient Number 10***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **14382/02** | **V** | C | Negative | D | H- | M- |
| **14382/02** | **IV** | Negative | Negative | Negative | H- | M- |
| **14382/02** | **III** | C | D | Negative | H- | M- |
| **14382/02** | **II** | C | D | D | H- | M+ |
| **14249/02** | **ssIII6** | A | A | A | H+ | M+ |
| **14249/02** | **ssIII4** | D | Negative | Negative | H- | M+ |
| **14249/02** | **ssIII5** | D | Negative | D | H+ | M+ |
| **14249/02** | **ssIII1** | D | Negative | D | H- | M- |
| **14249/02** | **ssIII2** | A | A | A | H+ | M+ |
| **14249/02** | **ssIII3** | A | A | A | H+ | M+ |
| **14249/02** | **ssIII7** | Negative | Negative | Negative | H- | M+ |

***Patient Number 11***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **26001/01** | **VII (1)** | A | A | A | H+ | M+ |
| **26001/01** | **VII (2)** | A | A | A | H+ | M+ |
| **26001/01** | **VII (3)** | A | A | A | H+ | M+ |

***Patient Number 12***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **9397/03** | **XII** | C | Negative | A | H- | M+ |
| **9397/03** | **VIII** | Negative | Negative | D | H- | M- |
| **9397/03** | **IX** | B | Negative | C | H- | M- |
| **9397/03** | **VI** | C | D | D | H- | M- |
| **9397/03** | **V** | C | Negative | Negative | H+ | M- |

***Patient Number 13***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **1570/03** | **VII** | A | A | A | H- | M+ |
| **1570/03** | **III(1)** | A | A | A | H+ | M+ |
| **1570/03** | **VI** | C | D | Negative | H- | M+ |

***Patient Number 14***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **16608/02** | **III (1)** | D | D | D | H- | M- |
| **16608/02** | **III (2)** | C | Negative | C | H- | M- |
| **16608/02** | **II** | C | Negative | C | H- | M+ |

***Patient Number 15***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **9409/02** | **I** | Negative | Negative | C | H- | M+ |

***Patient Number 16***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **6451/02** | **IV** | D | Negative | C | H- | M+ |
| **6451/02** | **V** | D | Negative | D | H- | M+ |

***Patient Number 17***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **30507/01** | **II** | C | Negative | C | H- | M+ |

***Patient Number 18***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **27887/01** | **ss** | Negative | B | C | H- | M+ |

| ***Patient Number*** | ***19*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **16438/03** | **III** | B | Negative | C | H- | M- |

| ***Patient Number*** | ***20*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **22778/02** | **I** | Negative | Negative | Negative | H- | M- |

| ***Patient Number*** | ***21*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **22509/02** | **IV** | Negative | Negative | Negative | H- | M- |

| ***Patient Number*** | ***22*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **15190/02** | **I** | Negative | D | C | H- | M- |

| ***Patient Number*** | ***23*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **14894/02** | **I** | Negative | Negative | C | H- | M- |

| ***Patient Number*** | ***24*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **14797/02** | **II** | Negative | Negative | Negative | H- | M- |

| ***Patient Number*** | ***25*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **8384/02** | **VI** | D | Negative | Negative | H- | M- |

| ***Patient Number*** | ***26*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **5669/02** | **I** | D | Negative | Negative | H- | M+ |

| ***Patient Number*** | ***27*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **79/02** | **ss(1)** | Negative | Negative | Negative | H- | M- |
| **79/02** | **ss(2)** | Negative | D | D | H- | M- |

| ***Patient Number*** | ***28*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **27744/01** | **V** | Negative | Negative | Negative | H- | M- |

***Patient Number 29***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **25807/01** | **II** | C | Negative | D | H- | M- |

***Patient Number 30***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **6189/02** | **VI** | C | D | Negative | H- | M- |

***Patient Number 31***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **10569/02** | **III** | B | D | D | H- | M- |

***Patient Number 32***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **12002/02** | **Vss** | Negative | D | D | H- | M- |

***Patient Number 33***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **13273/02** | **II** | C | Negative | Negative | H- | M- |

***Patient Number 34***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **4217/03** | **ssIII** | Negative | Negative | D | H- | M- |

***Patient Number 35***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **15824/03** | **VI** | C | Negative | D | H- | M- |
| **15681/03** | **ssII** | Negative | Negative | Negative | H- | M- |
| **15680/03** | **ssIV** | C | C | C | H- | M- |
| **15680/03** | **ssIII** | Negative | Negative | Negative | H- | M- |
| **15680/03** | **ssI** | C | Negative | D | H- | M- |
| **15680/03** | **ssII** | C | Negative | C | H- | M- |

***Patient Number 36***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **28011/03** | **ss(3)** | A | A | A | H+ | M+ |
| **28011/03** | **ss(2)** | A | A | A | H+ | M+ |
| **28012/03** | **ss(1)** | A | A | A | H+ | M+ |
| **28012/03** | **ss(2)** | A | A | A | H+ | M+ |
| **28011/03** | **ss(1)** | A | A | A | H+ | M+ |

***Patient Number 37***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **30836/03** | **ssI** | A | A | A | H- | M- |
| **30836/03** | **ssII** | A | A | A | H+ | M- |
| **30836/03** | **ssIII** | D | D | D | H- | M- |
| **30836/03** | **ssIV** | D | D | C | H- | M- |

***Patient Number 38***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **29524/03** | **IX** | B | D | B | H- | M- |
| **29524/03** | **XII** | C | D | Negative | H- | M+ |
| **29524/03** | **X** | C | Negative | D | H- | M- |
| **29524/03** | **VIII** | C | D | D | H- | M+ |
| **29524/03** | **XI** | C | Negative | Negative | H- | M- |

***Patient Number 39***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **4479/04** | **IV** | D | D | C | H- | M+ |
| **4479/04** | **V** | D | Negative | C | H- | M+ |
| **4479/04** | **VI** | C | D | C | H- | M+ |
| **4459/04** | **ss II** | A | A | A | H- | M+ |

***Patient Number 40***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **26134/03** | **IIIss** | Negative | Negative | Negative | H- | M- |
| **26134/03** | **Ivss** | Negative | Negative | Negative | H- | M- |
| **26134/03** | **Iss** | Negative | D | D | H- | M- |
| **26134/03** | **IIss** | A | Negative | A | H+ | M+ |
| **26134/03** | **Vss** | C | Negative | C | H- | M- |

***Patient Number 41***

| *Histo No* | *SLN* | *p16* | *CK19* | *AE 1/3* | *Histology* | *HPV mRNA* |
|---|---|---|---|---|---|---|
| **23301** | **III** | B | Negative | D | H- | M- |
| **23222/03** | **ssIII** | Negative | Negative | D | H- | M- |
| **23222/03** | **II2.Ber** | D | D | Negative | H- | M+ |
| **23224/03** | **ssI** | A | A | A | H- | M+ |
| **23224/03** | **ssII** | Negative | Negative | D | H+ | M- |
| **23224/03** | **II2.Ber** | Negative | Negative | Negative | H+ | M- |

| ***Patient Number*** | ***42*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **12992/02** | **III** | B | Negative | C | H- | M+ |

| ***Patient Number*** | ***43*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **30487/02** | **III** | C | Negative | Negative | H- | M- |
| **30487/02** | **V** | C | D | C | H- | M+ |
| **30487/02** | **IV** | C | D | A | H- | M- |

| ***Patient Number*** | ***44*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **14697/01** | **V** | C | C | C | H- | M+ |
| **14697/01** | **II** | C | C | D | H- | M+ |

| ***Patient Number*** | ***45*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **24373/01** | **III** | A | A | A | H+ | M+ |

| ***Patient Number*** | ***46*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **22208/01** | **VI** | Negative | Negative | Negative | H- | M+ |
| **22208/01** | **XII** | Negative | Negative | C | H- | M+ |

| ***Patient Number*** | ***47*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **19673/01** | **V** | A | A | A | H+ | M+ |

| ***Patient Number*** | ***48*** | | | | | |
|---|---|---|---|---|---|---|
| ***Histo No*** | ***SLN*** | ***p16*** | ***CK19*** | ***AE 1/3*** | ***Histology*** | ***HPV mRNA*** |
| **17891/01** | **VI** | B | C | C | H- | M- |

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# Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsprüfungsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist, ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind, mich Herr Prof. Dr. Dürst bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt hat, ich die Hilfe eines Promotionsberaters nicht in Anspruch genommen habe, dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

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